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Title:

Epi-eDNA: From Methylation Signal Detection to Functional Ecological Monitoring

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Abstract (< 100 words):

Environmental DNA (eDNA) technology has revolutionized biomonitoring, primarily capturing the presence/absence of target taxa. Recent advances have revealed that eDNA also retains epigenetic signatures (epi-eDNA), particularly DNA methylation, which enable functional ecological insights. This review synthesizes three pivotal milestones: (1) Initial detection of methylation signals in eDNA, confirming the feasibility of the epi-eDNA concept, (2) verification of stability across environmental matrices, demonstrating fidelity to source-tissue profiles despite degradation, and (3) emerging applications as ecological indicators—using epigenetic clocks for age-structure assessment, sex-specific markers for population sex ratios, germ cells methylation for spawning detection, and stress-linked methylation for health monitoring. This review highlights the potential of epi-eDNA in non-invasive population-level trait inference, overcoming the limitations of traditional eDNA. Future integration with multi-omics and sequencing innovations will unlock unprecedented precision in conservation and ecosystem management.

Main text (< 3000 words; currently 5,139 words):

Introduction

Environmental DNA (eDNA) refers to genetic material obtained directly from environmental samples, such as water, soil, and air (Banchi *et al.* 2020; Bohmann *et al.* 2014; Pawlowski *et al.* 2020). As organisms interact with their surroundings, they continuously release eDNA into the environment, which accumulates in various forms (Power *et al.* 2023). eDNA may include freely available DNA or may originate from cellular debris shed during movement or activity, such as hair, feathers, scales, setae, molted skin, feces, active and dormant plant tissues, seeds, pollen, and plant fragments (Fahner *et al.* 2016; Valentin *et al.* 2018; Cavill *et al.* 2022). A typical eDNA-based biodiversity monitoring starts with collecting environmental samples (e.g., water, soil, or air), followed by eDNA extraction. The downstream workflow then diverges depending on the method used. Currently, qPCR and eDNA metabarcoding are commonly utilized for eDNA-based biodiversity monitoring, whereas shotgun metagenomics is emerging. qPCR amplifies species-specific genetic markers, enabling the detection and quantification of known target species eDNA (Hernandez *et al.* 2020; Benoit *et al.* 2023; Guri *et al.* 2024). eDNA metabarcoding involves the amplification of taxonomically informative marker genes, high-throughput sequencing, and subsequent taxonomic classification to infer the presence of species belonging to a specific taxon, such as fish (Lacoursière-Roussel *et al.* 2018; Bylemans *et al.* 2019; Ruppert *et al.* 2019; Miya *et al.* 2015). In contrast, shotgun metagenomic sequencing enables unbiased analysis of eDNA by randomly sequencing all DNA fragments in a sample, avoiding the PCR biases of metabarcoding while revealing community composition, which has the potential to be another primary method to analyze eDNA (Tessler *et al.* 2017; Cowart *et al.* 2018; Manu & Umapathy 2023; McCauley *et al.* 2024; Nousias *et al.* 2025).

One of the main advantages of using eDNA in biomonitoring programs is the relative ease of sample collection under field conditions and its non-invasive nature. In aquatic monitoring, for instance, sampling, filtration, and preservation can typically be completed in a short time. Compared to traditional monitoring techniques such as seining (Dias *et al.* 2022), electrofishing (Thomas *et al.* 2019), and visual observation (e.g., for marine mammals; Urian et al. 2015), sample collection for eDNA analysis is more time-efficient (d'Auriac *et al.* 2019; Deiner *et al.* 2016). In addition, water samples can be collected without causing any noticeable disturbance to the environment (Carraro *et al.* 2018). The low time- and labor-cost for sampling and the non-invasive nature enable researchers to perform more frequent and broader sampling, improving the ability to detect early invasive species (Furlan *et al.* 2016) and assessing temporal changes in community composition (Rozanski *et al.* 2024; Ushio 2022; Ushio *et al.* 2023).

Nonetheless, although they are effective for biodiversity monitoring, the information that eDNA analysis can provide is still relatively limited to presence/absence detection (Beauclerc *et al.* 2019; Davison *et al.* 2019), species identification (Klymus *et al.* 2017; Mächler *et al.* 2019), and abundance estimation (Lacoursière-Roussel *et al.* 2016; Yates *et al.* 2021; but see Harrison *et al.* 2021). Recently, new eDNA techniques have been developed to detect more detailed information about biodiversity. For example, intra-specific diversity (Mächler *et al.* 2019; Tsuji *et al.* 2023; Zanovello *et al.* 2024), community composition and functional potential through metagenomics (Cowart *et al.* 2018; Ragot & Villemur 2022), and gene

expression activity through environmental RNA (Seeber & Epp 2022; Li *et al.* 2024a). Among these new directions, how to detect and utilize epigenetic information (e.g., DNA methylation) is a recent frontier in eDNA research.

Epigenetics is the study of heritable changes in gene expression that occur without alterations to the underlying DNA nucleotide sequence (Egger *et al.* 2004), including transcription factors, noncoding RNAs, DNA methylation, and histone modifications (Portela & Esteller 2010). DNA methylation refers to the covalent addition of a methyl group ($–CH_3$) to the 5th carbon of the cytosine ring in DNA, forming 5-methylcytosine (5mC), primarily at cytosine-phosphate-guanine (CpG) dinucleotides (Field *et al.* 2018; Smith *et al.* 2025). DNA methylation is regulated by DNA methyltransferase enzymes (DNMTs). DNMT1 is responsible for maintaining existing methylation patterns during DNA replication, while DNMT3A and DNMT3B establish *de novo* methylation at new loci (Kulis & Esteller 2010). Moreover, methylation is reversible. Active demethylation is mediated by TET family enzymes (TET1, TET2, and TET3), which oxidize 5mC to 5-hydroxymethylcytosine (5hmC), and subsequently to other forms that are ultimately processed back to unmethylated cytosine through base excision repair (Wu & Zhang 2017). Genomic regions with a high frequency of CpG dinucleotides, called "CpG islands", that are typically GC-rich and frequently located in or near gene promoter regions (Issa 2004), which often serve as a target of DNA methylation studies. The biological roles of DNA methylation include silencing gene expression (especially promoter hypermethylation inhibits transcription); X-chromosome inactivation; genomic imprinting; repression of repetitive elements; and maintenance of genomic stability (Jones *et al.* 2015; Smith *et al.* 2025).

Previous research has shown that methylation landscapes exhibit high cell-type specificity and tissue dependence, making them powerful signals for cellular identity and phenotype (Stubbs *et al.* 2017; Lau & Robinson 2021). DNA methylation plays a pivotal role in the phenotypic prediction and mechanistic understanding of various human diseases, particularly cancers (e.g., Nishiyama & Nakanishi 2021), cardiovascular disease (e.g., Krolevets *et al.* 2023), and autoimmune syndromes (e.g., Lafontaine *et al.* 2023). Importantly, DNA methylation patterns also serve as powerful signals of age, as they reflect both programmed biological processes and the stochastic accumulation of changes over time. Specific CpG sites (Horvath 2013; McEwen *et al.* 2020) undergo highly predictable, often linear, methylation changes that are tightly correlated with chronological age across the lifespan, likely reflecting underlying developmental and maintenance programs. Moreover, DNA methylation patterns exhibit striking sex-specific differences that are detectable across tissues, developmental stages, and species. Numerous CpG sites, particularly on the X chromosome but also on autosomes, display consistent sex-associated methylation patterns from birth onward (Liu *et al.* 2010; Yousefi *et al.* 2015), although X-chromosome inactivation (Lyonization) is restricted to mammals and some rare exceptions (e.g., particular aphid species). These epigenetic signatures are implicated in shaping sex-biased gene expression and contribute to developmental, metabolic, and disease-related dimorphisms (Davegårdh *et al.* 2019; Govender *et al.* 2022). The role of DNA methylation in the reproductive process is primarily reflected in regulating gene expression during gonad development (Li *et al.* 2019), responding to environmental change signals (Venney *et al.* 2022; Saito *et al.* 2025), and thus regulating germ cell formation (Laing *et al.* 2018).

Especially before and after spawning, dynamic changes in methylation status determine whether reproductive function is activated or suppressed (Woods III *et al.* 2018; Nilsson *et al.* 2021). This mechanism is not only crucial for individual physiological regulation but also has wide application value in ecological adaptation and species protection.

Detecting epigenetic signals of eDNA (epi-eDNA) has the potential to overcome several limitations of conventional eDNA-based biodiversity monitoring (Balard *et al.* 2024; Fig. 1). The term was first introduced by Schadewell & Adams (2021) to describe eDNA that retains epigenetic signals. In this review, we adopt a broad definition of eDNA, which encompasses DNA obtained not only from environmental substrates such as water, soil, and air but also from other biological materials found in the environment, including feces and shed tissues. This inclusive definition highlights the diversity of DNA sources used for biodiversity monitoring. Additionally, we use epi-eDNA specifically to refer to eDNA fragments—under our broad definition of eDNA—that preserve epigenetic modifications, and we particularly focused on (e)DNA methylation in this review. Such modifications provide additional biological information beyond the primary nucleotide sequence typically analyzed in conventional eDNA studies.

Our review will introduce (1) how to measure DNA methylation rates, (2) existing examples of the detection of DNA methylation signals in eDNA, and (3) potential applications of epi-eDNA.

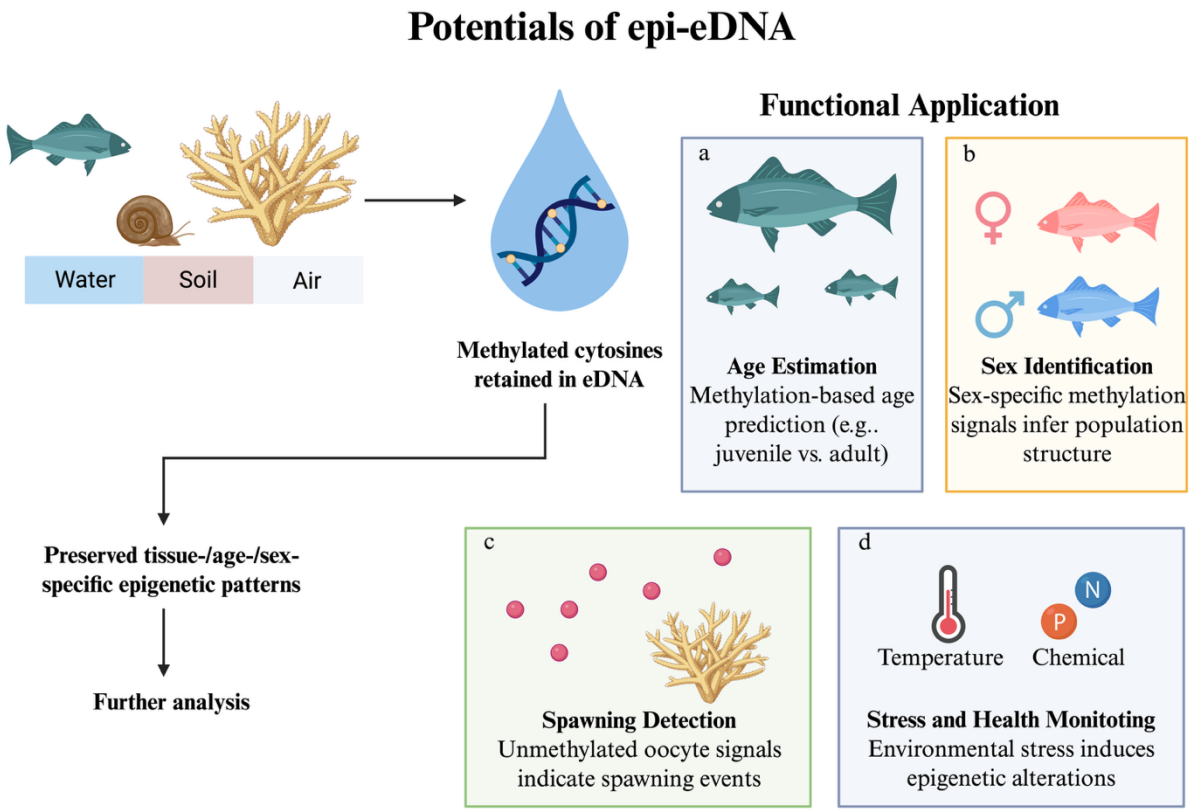

Figure 1. Potentials of epi-eDNA to detect detailed characteristics of target organisms from eDNA.

There are four key ecological applications of epi-eDNA: (a) Age structure assessment through methylation-based epigenetic clocks; (b) Sex ratio estimation via sex-specific methylation signals; (c) Spawning detection by identifying germ cell-derived demethylation signatures; and (d) Stress and health monitoring. These advances transform eDNA from a taxonomic census tool into a functional phenotyping platform, revealing mechanisms underlying biodiversity dynamics such as reproductive timing, demographic shifts, and stress responses.

Methods for measuring DNA methylation either in tissue-derived DNA or eDNA

The rapid development of DNA methylation research has been closely tied to continuous innovation in both molecular detection platforms and computational modeling strategies. From data acquisition to biological interpretation, these technical infrastructures form a vital bridge connecting methylome information with diverse phenotypic traits. Currently, the most widely adopted DNA methylation profiling techniques include methylation-specific polymerase chain reaction (MSP), whole-genome bisulfite sequencing (WGBS), reduced representation bisulfite sequencing (RRBS), targeted bisulfite sequencing including bisulfite amplicon sequencing (BSAS) and pyrosequencing, site-specific techniques including methylation-sensitive high-resolution melting (MS-HRM) and 5-Methylcytosine binding domain (MBD) sequencing (Licchesi & Herman 2009; Masser *et al.* 2015; Català *et al.* 2015; Hussmann & Hansen 2018; Dhingra *et al.* 2019; Jeltsch *et al.* 2020; Beck *et al.* 2022). Basically, methods for quantifying DNA methylation can be categorized along two axes: chemistry (bisulfite vs. non-bisulfite) and scope (genome-wide vs. locus-specific). We will introduce them in the following sections.

Bisulfite-based methods

Bisulfite-based methods are one of the most widely used, which include reactions of unmethylated cytosine (C) converted to uracil (U) by sodium bisulfite. Methylated cytosines, however, are protected from this conversion and remain unchanged. Following bisulfite treatment, the DNA methylation patterns can be analyzed using sequencing-based methods or PCR-based methods. Using sequencing-based methods, researchers can identify which cytosines are methylated by aligning the bisulfite-converted sequence data with a reference genome (Ogino *et al.* 2006). Among genome-wide bisulfite methods, WGBS is often considered the gold standard, offering single-base resolution across the entire genome, which enables mechanistic and comprehensive mapping (Beck *et al.* 2022; Habibi *et al.* 2013). WGBS, despite its resolution, is often cost-prohibitive for huge cohorts due to high per-sample sequencing depth and downstream compute needs. WGBS is generally challenging to conduct for studies using large sample sizes because of its high cost per sample. RRBS is more cost-effective and focused on CpG-rich regions, making it suitable for studies on transcriptional regulation (Ma *et al.* 2022; El Kamouh *et al.* 2024). However, it

offers broad but sparse genomic representation, with limited CpG enrichment and low reproducibility across biological replicates.

Bisulfite-based methods also include several locus-specific assays, notably MSP, BSAS, pyrosequencing, and MS-HRM. MSP is performed using primers specifically designed to match either the bisulfite-modified methylated or unmethylated sequences, thereby selectively amplifying either methylated or unmethylated sequences. This allows for the determination of the methylation status at target CpG sites or regions (Licchesi & Herman 2009). Its advantages include high sensitivity due to PCR-based discrimination and a simple workflow adaptable to low-input or degraded samples via nested or multiplex MSP. At the same time, its limitations lie in the dependence on proper primer design and complete bisulfite conversion, susceptibility to PCR contamination and bias, and limited ability to detect subtle methylation differences (Licchesi & Herman 2009). BSAS integrates next-generation sequencing with a library preparation that includes locus-specific PCR enrichment after bisulfite conversion (Masser *et al.* 2015). BSAS offers high sensitivity and cost-efficiency for multi-gene parallel DNA methylation analysis. Still, it is limited by poor coverage of large CpG islands and potential bias from heterogeneous tissue sources (Moser *et al.* 2020).

Bisulfite-based pyrosequencing is also commonly used in the development of epigenetic clocks for cetaceans and other mammals (Polanowski *et al.* 2014; Beal *et al.* 2019; Nakamura *et al.* 2023a). Pyrosequencing is a method that detects real-time light signals from the release of pyrophosphate during nucleotide incorporation. Pyrosequencing accurately quantifies DNA methylation at specific CpG sites, making it a gold standard for regional methylation analysis (Kumar *et al.* 2020).

Another widely utilized technique is PCR-based methods, such as MS-HRM, which has high sensitivity for detecting DNA methylation at specific loci (Hussmann & Hansen 2018). Rather than directly sequencing bisulfite-converted DNA, MS-HRM employs high-resolution melting analysis to detect methylation-induced sequence alterations. This method is particularly suitable for screening applications and low-frequency methylation detection with minimal cost (Qi *et al.* 2021). Its special primer design strategy, which ensures equal amplification efficiency for both methylated and unmethylated templates, can minimize PCR bias and allow the detection of rare methylation events at frequencies as low as 0.1% (Hussmann & Hansen 2018). The limitation of MS-HRM is that methylation levels are not provided per CpG site, but only as an average across the amplified region.

Non-bisulfite methods

Among non-bisulfite methods, methyl-CpG-binding domain (MBD)-based methods are widely used for genome-wide scale studies. Due to its methylation-specific DNA binding capacity, MBD can bind methylated DNA in vitro, thereby supporting genome-wide

methylation profiling (Jeltsch *et al.* 2020). MBD sequencing supports DNA methylation profiling by using methyl-CpG-binding proteins to enrich methylated fragments from sheared and purified genomic DNA, followed by next-generation sequencing and bioinformatic workflows. It was demonstrated that MBD-seq is a cost-effective method for obtaining genome-wide CpG methylation information, particularly suitable for large-scale research in fish breeding and nutrition (Naya-Català *et al.* 2023).

Additionally, a nanopore sequencing-based protocol was developed to detect base modifications in eDNA molecules derived from water samples (Ruiz *et al.* 2025). Nanopore sequencing is a long-read DNA sequencing technology developed by Oxford Nanopore Technologies (ONT). It detects DNA sequences by measuring changes in electrical current as DNA molecules pass through a nanopore. This method can directly identify epigenetic modifications without chemical conversion or amplification (Doshi *et al.* 2025). Depending on the library preparation and enrichment, it supports both genome-wide methylation profiling and locus-specific analysis (Flynn *et al.* 2022; Ruiz *et al.* 2025).

Lastly, PacBio-based methylation profiling is another method for detecting DNA methylation using Pacific Biosciences Single Molecule Real-Time (SMRT) sequencing. It identifies methylated bases by analyzing real-time kinetic signatures that are generated during DNA synthesis. This method does not require chemical treatment or bisulfite conversion and enables base-resolution, quantitative, and strand-specific methylation detection directly from native DNA molecules (Somerville *et al.* 2019). The approach can be applied genome-wide using whole-genome sequencing (Somerville *et al.* 2019), or locus-specifically using PCR-free CRISPR–Cas9 (Tsai *et al.* 2017), enabling both comprehensive surveys and focused interrogation of selected regions. A recent technology, named enzymatic methyl-seq (EM-seq), is a bisulfite-free, enzyme-based method for detecting 5mC and 5hmC at single-base resolution (Vaisvila *et al.* 2021). During PCR amplification, unmethylated cytosine (C) is read as thymine (T), while 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) remain as cytosine (C), thus achieving high differentiation. EM-seq offers high sensitivity, even coverage, and DNA integrity with low input requirements; however, it cannot detect 5fC/5caC and involves complex enzymatic steps.

The accompanying Table 1 is organized based on two criteria (non-bisulfite-based vs. bisulfite-based; genome-wide vs. locus-specific) to elucidate the methodological landscape for eDNA methylation detection.

Table 1. Pros and Cons of Different Methods for Studying DNA Methylation.

Methods	Method Type	Resolution Scope	Pros	Cons	Reference
Whole-Genome Bisulfite	Bisulfite	Genome-wide	- Single-base resolution	- Expensive - Requires high	Beck *et al.* (2022)

Sequencing (WGBS)			across entire genome - Ideal for comprehensive methylation mapping	input DNA - Computationally intensive	
Reduced Representation Bisulfite Sequencing (RRBS)	Bisulfite	Genome-wide	- Cost-effective - Enriches CpG-rich regions - Suitable for transcriptional regulation studies	- Limited genome coverage - Bias towards certain regions	El Kamouh *et al.* (2024)
Methylation-specific polymerase chain reaction (MSP)	Bisulfite	Loci-specific	- High sensitivity - Works on low-quantity or low-quality DNA and allows multiple promoters to be profiled - Technically easy	- Highly dependent on primer design and complete bisulfite conversion - Susceptible to PCR cross-contamination and bias - Limited to detect minor methylation differences	Licchesi & Herman (2009)
Bisulfite Amplicon Sequencing (BSAS)	Bisulfite	Loci-specific	- Scalable for multiple loci - Integrates NGS with locus-specific PCR	- Requires sequencing infrastructure - Limited to predefined regions	Masser *et al.* (2015)

			- Efficient for targeted analysis		
Methylation-Sensitive High-Resolution Melting (MS-HRM)	Bisulfite	Loci-specific	- High sensitivity (detects ~0.1% methylation) - Low cost - PCR-based, simple setup - Suited for rare event detection	- Semi-quantitative - Interpretation may be complex - Requires careful primer design - Does not provide methylation levels per CpG and only leads an average methylation level across the amplified region.	Hussmann & Hansen (2018)
5-methylcytosine binding domain (MBD)	Non-bisulfite	Genome-wide	- Low-cost dsDNA enrichment - Reliable (high cross-assay concordance) - 5-mC-specific	- Medium resolution - CpG-density bias (preferentially captures CpG-rich, densely methylated regions) - Detects 5-mC only (does not directly report 5-hmC)	Jeltsch *et al.* (2020)
Nanopore-based epi-eDNA profiling	Non-bisulfite	Genome-wide & Loci-specif	- Detects methylation directly	- Relatively new - May have lower accuracy	Doshi *et al.* (2025)9/11/25

		ic	- No need for bisulfite conversion or amplification - Suitable for eDNA from water samples	than bisulfite-based methods	12:17:00 PM
PacBio-based methylation profiling	Non-bisulfite	Genome-wide & Loci-specific	- Detects native methylation (e.g., 5mC, 6mA) directly during sequencing - No bisulfite conversion or amplification needed - Enables complete genome assembly	- High raw error rate (requires polishing) - Expensive and requires high DNA input - Challenging for high-complexity microbiomes	Somerville *et al.* (2019)
Enzymatic Methyl-seq (EM-seq)	Non-bisulfite	Genome-wide	- Non-destructive to DNA - High CpG detection - Effective with low-input and degraded	- Does not detect 5fC or 5caC - Dependent on precise enzyme activity and reaction conditions - Slightly reduced	Vaisvila *et al.* (2021)

			samples -	detection in highly methylated DNA	

Detection of DNA methylation signals in aquatic environmental DNA

Traditionally, eDNA has been utilized primarily for species detection (Rees *et al.* 2014; Sigsgaard *et al*. 2019). However, eDNA analysis could be utilized to explore population genetics (Adams *et al.* 2019) and even population epigenetics (Balard *et al.* 2024). Building on this conceptual expansion, researchers started to explore whether DNA methylation signals can be preserved in eDNA. In this section, we will introduce recent studies that detected DNA methylation signals in eDNA.

Freshly shed eDNA likely retains the methylation signals of the source organism, though these methylated cytosines are prone to deamination as the DNA degrades (Sigsgaard *et al.* 2019). This hypothesis laid a theoretical foundation for detecting epigenetic signals in environmental samples. Following this, the years 2022–2023 saw the emergence of the first empirical studies that detected DNA methylation signals in aquatic systems (mainly in aquarium tanks). For example, Zhao *et al*. (2023) conducted aquarium tank experiments using the freshwater snail (*Lymnaea stagnalis*), comparing methylation profiles between tissue-derived DNA and eDNA extracted from water samples. Tissue DNA was extracted from four life stages of snails, and eDNA was extracted from tanks with the four life stages of snails, which were sequenced to assess methylation levels. Water eDNA exhibited clear and quantifiable DNA methylation patterns, which varied significantly across water eDNA extracted from tanks with snails of different developmental stages. This study provided the first experimental evidence that water eDNA carries information on population-level traits such as age structure.

Zhao *et al*. (2023) demonstrated that DNA methylation can be detected in water eDNA; however, it remains unclear whether these signals are stably retained in environmental samples and accurately reflect the methylation status of the source tissue. Recent studies have provided some evidence. For example, Hirayama *et al*. (2024) conducted a tank experiment focused on the zebrafish species (*Danio rerio*) and demonstrated that even after partial degradation of eDNA in the environment, the methylation levels of eDNA closely mirrored those of the original somatic tissue DNA. Additionally, they found no significant differences in methylation rates between tissue-derived DNA and eDNA. Furthermore, the study also investigated the effects of different cellular sources on the methylation signals. During the peak spawning period of fish, the water eDNA showed a high abundance of unmethylated DNA fragments, which could originate from germ cells. The gene regions they detected are

known to be highly unmethylated in maternal germ cells and were possibly released into the aquatic environment during oviposition, supporting the idea that not only global methylation levels, but also cell–type–specific methylation signatures can be retained in eDNA.

Potential applications of epi-eDNA to ecological studies

Based on some pioneering studies that detected DNA methylation signals in environmental samples, researchers have begun exploring the use of eDNA methylation to infer functional ecological information. Epi-eDNA, like eDNA, provides information at the population level rather than the individual level because eDNA typically represents a mixture of DNA from multiple individuals. As a result, the detected epigenetic signals represent an aggregate of biological traits across the population. Since epigenetic signals are closely associated with biological characteristics such as age, sex, reproductive state, and health status, epi-eDNA is a promising tool for monitoring population-level functional attributes. Here, we introduce several potential applications of epi-eDNA to study ecological communities, namely, (1) age structure inference, (2) sex identification and sex ratio monitoring, (3) reproductive state and spawning detection, and (4) health status and stress responses.

Age structure

DNA methylation changes predictably with chronological age, enabling the development of epigenetic clocks—statistical models that estimate biological age from a subset of CpG methylation levels (Teschendorff & Horvath 2025).

The pan-tissue epigenetic clock proposed by Horvath (2013) demonstrated high accuracy in methylation-based age prediction across cell types and tissues in humans. For non-human species, minimally invasive epigenetic approaches have been developed. Polanowski et al. (2014) was the first study to establish an epigenetic clock for cetaceans by targeting specific loci using pyrosequencing. It was also the first to investigate which loci exhibit age-associated DNA methylation, identifying three key loci, *TET2*, *GRIA2*, and *CDKN2A*. This foundational work laid the groundwork for subsequent research, including the study by Beal *et al.* (2019). The Bottlenose Dolphin Epigenetic Aging Tool (BEAT) utilizes DNA methylation levels in skin biopsy samples for age estimation in small cetaceans (Beal *et al.* 2019). Similarly, Mori *et al.* (2024) established a non-lethal method to estimate the chronological age of Risso's dolphins (*Grampus griseus*) by analyzing DNA methylation in skin tissues, providing an alternative to traditional tooth growth layer group (GLG) counting. A blood DNA methylation-based age prediction model was also developed for brown bears (*Ursus arctos*) across populations, offering a practical tool for wildlife conservation (Nakamura *et al.* 2023). Lu *et al.* (2023) showed a universal mammalian epigenetic clock capable of accurately predicting tissue age across diverse mammalian species and tissue types.

They compiled the results of 11754 methylation arrays (a microarray that measures the level of methylation at specific CpG sites) from 185 species. They presented three models using different age indices: a basic chronological age clock, a relative age clock (chronological age divided by maximum lifespan), and a log-linear clock (utilizing age at sexual maturity and gestation time). All models demonstrated high prediction accuracy, with a correlation coefficient (r) exceeding 0.95 (for example, see Fig. 2a for bottlenose dolphins).

Estimating population age composition is one of the early applications of epi-eDNA. Other than the study on the freshwater snail (Zhao *et al.* 2023), Ruiz *et al.* (2025) developed a genome-wide methylation "epigenetic clock" using eDNA derived from aquaculture tanks of European seabass (*Dicentrarchus labrax*). Their model achieved a median age prediction error of only 2.6 days for larvae aged 10–24 days, comparable to or exceeding the accuracy of traditional tissue-based methods such as otolith or scale analysis. This result validates the feasibility of using epi-eDNA for high-accuracy, non-invasive age estimation in wild populations. In addition, Yagi *et al.* (2024) demonstrated that methylation clocks from fecal samples of wild Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) using MS-HRM analysis targeting *GRIA2/CDKN2A* achieved an accuracy of MAE of 5.08 years (10–13% of lifespan; Fig. 2b). Also, Hanski *et al.* (2024) established an epigenetic clock for house mice (*Mus musculus*) based on DNA extracted from fecal samples. While the clock, trained on laboratory mice, yielded highly accurate age predictions both in the training set (mean absolute error (MAE) = 23 days; Fig. 2c) and in the validation set (MAE = 26 days), its application to wild mice revealed individual variation in epigenetic aging rates. Despite these differences, the clock successfully distinguished juveniles from adults in natural populations of house mice, marking one of the first demonstrations of fecal epi-eDNA for age inference in wild mammals.

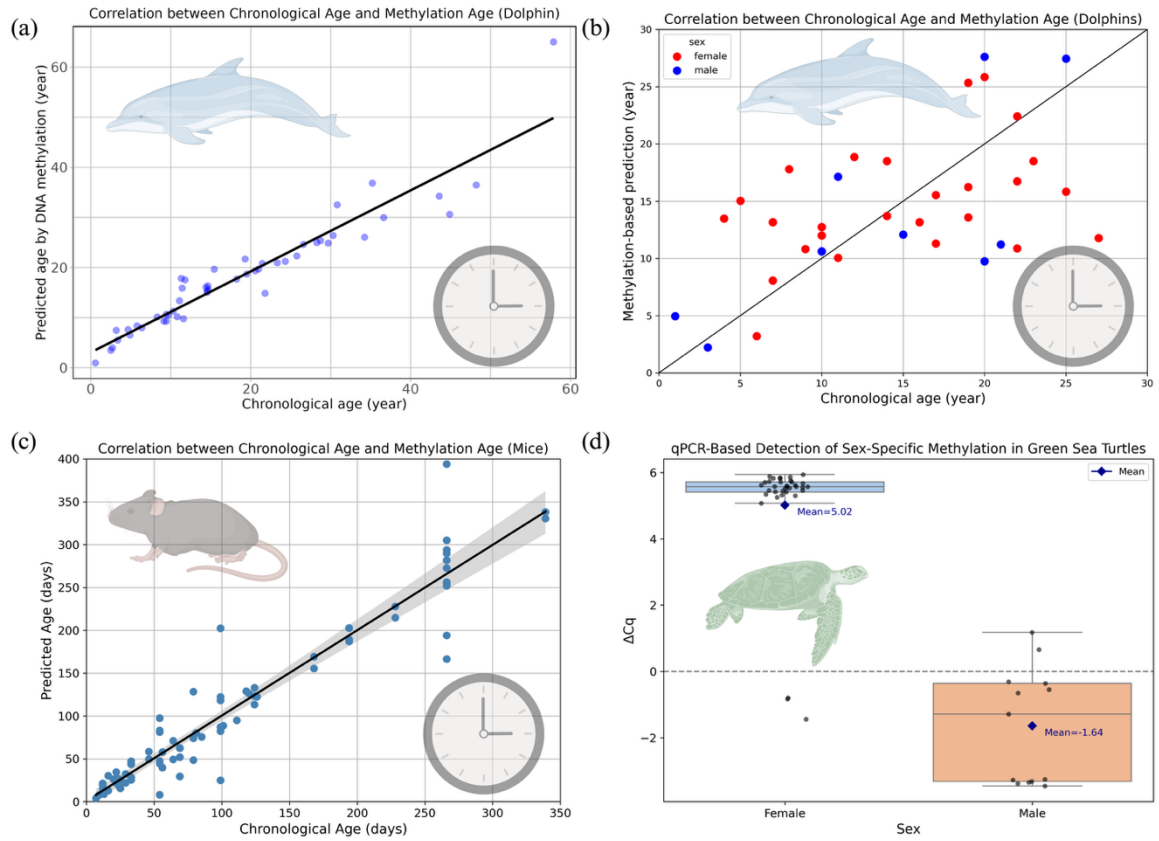

Figure 2. Applications of DNA methylation to infer the age and sex of target organisms. Some of the key applications of DNA methylation include inferring age and sex. (a) An epigenetic clock based on skin tissue DNA in bottlenose dolphins (*Tursiops truncatus*) (recreated using the data from Lu *et al*. 2023). (b) Age prediction in wild Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) using fecal epi-eDNA. The scatter plot shows the predicted versus actual age, colored by sex (red for females and blue for males), recreated using data from Yagi *et al*. (2024). (c) Age estimation in wild house mice (*Mus musculus*) using faecal epi-eDNA (recreated using the data from Hanski *et al*. 2024). (d) Detection of sex-specific methylation differences in skin tissue of green sea turtles (*Chelonia mydas*) using qPCR targeting the *UBN2* gene. A positive ΔCq was suggested to be a female, while a negative value is a male (recreated using the data from Mayne *et al*. 2023). Created in BioRender. Chengbin, L. (2025) https://BioRender.com/j9pl83z

Sex identification and sex ratio monitoring

Sex-specific DNA methylation is a fundamental epigenetic mechanism that underlies sexual differentiation across a wide range of animal species. In mammals, such as mice, genome-wide methylation studies have revealed sexually dimorphic patterns in tissues like the liver, with differential methylation regions (DMRs) influenced not only by genetic background but also by physiological experiences such as pregnancy, indicating the persistence of epigenetic memory linked to sex and life history events (Grimm *et al.* 2019).

Fish exhibit remarkable sexual plasticity, with numerous species undergoing natural sex transitions during their lifetime or experiencing sex reversal in response to environmental factors, such as temperature, social cues, and stress. Epigenetic mechanisms, particularly DNA methylation and histone modifications, play pivotal roles in both initiating and stabilizing these sex transitions. For sex identification, epigenetic approaches are especially valuable because they capture phenotypic states rather than relying exclusively on genotypic information. Given the high diversity and plasticity of sex in fish, methodologies that do not rely on genotyping are critical in eDNA analysis, where direct observation of individuals is unfeasible (Ortega-Recalde *et al.* 2020).

DNA methylation plays a pivotal role in sex determination and transition in zebrafish, which lack heteromorphic sex chromosomes; reprogramming events in primordial germ cells (PGCs) mirror those of oocytes during early development, and inhibiting DNA methylation prevents female-to-male transition, establishing a causal role for methylation in sexual fate (Wang *et al.* 2021). Temperature-sensitive species like the American alligator (*Alligator mississippiensis*) and the tiger pufferfish (*Takifugu rubripes*), whose sex is determined by incubation temperature during the critical developmental phase, demonstrate that environmental cues can induce sex-specific methylation reprogramming, enable accurate prediction of hatchling sex, or facilitate sex reversal via methylation changes in key developmental genes such as *amhr2* and *cyp19a* (Sun *et al.* 2022; Zhou *et al.* 2019). Even in insects like aphids (*Myzus persicae*), where males are haploid for the X chromosome, methylation patterns compensate for gene dosage, with X-linked genes hypermethylated and autosomal genes hypomethylated in males, suggesting a conserved mechanism of dosage regulation through epigenetics (Mathers *et al.* 2019). In hermaphroditic *Argopecten* scallops, sterile hybrids show significantly higher global methylation levels than fertile parents, with differentially methylated genes (DMGs) enriched in pathways like ubiquitin-mediated proteolysis and ECM-receptor interaction, suggesting impaired oogenesis due to ROS accumulation and ATP deficiency (Yu *et al.* 2023). Likewise, studies in Pacific oysters (*Crassostrea gigas*), an invertebrate species lacking defined sex chromosomes, reveal that male gonads exhibit significantly higher methylation levels than females. That sex-biased methylation is enriched in broadly expressed regulatory genes rather than canonical sex-determining loci, pointing to a distributed epigenetic architecture (Sun *et al.* 2022). Altogether, these findings suggest that sex-associated DNA methylation is flexible and responsive to environmental and developmental contexts, making it a critical regulator of sexual dimorphism in animal biology.

Although no published studies have used epi-eDNA for population-level sex ratio inference, the concept is theoretically established. Many organisms exhibit sex-specific methylation patterns across the genome (Bock *et al.* 2022; Chien *et al.* 2024). If DNA from sexually dimorphic loci is released and retained in environmental matrices, it should be

possible to infer sex ratios based on the methylation status of eDNA. In Zhao *et al*. (2023), which detected the methylation rate of eDNA from freshwater snail, a hermaphroditic species was chosen to avoid confounding effects from sex-specific methylation. It was noted that for gonochoristic species, sexually dimorphic methylation could be a target signal. Many reptiles, including turtles, exhibit temperature-dependent sex determination (TSD). In such species, genotyping alone cannot be used to reliably identify phenotypic sex (While *et al.* 2018). Indeed, studies on green sea turtles have successfully utilized methylation profiles from skin tissue to determine sex (Mayne *et al.* 2023). Mayne *et al*. (2023) applied RRBS to adult green turtle skin biopsies to pinpoint 16 sex-specific methylation loci, then built methylation-sensitive qPCR assays that achieve 100 % accuracy in sexing turtles older than 17 years. It provides a rapid, less invasive, and cost-effective tool for determining the sex of adult green turtles. These findings lay a conceptual and methodological foundation for the future use of epi-eDNA in sex ratio estimation.

Reproductive state and spawning detection

DNA methylation regulates gene expression during reproduction, affecting gonadal development, sex determination, hormonal signaling, fertility, and spawning timing. Dynamic, stage-specific methylation patterns have been observed in gametogenesis across species, such as scallops (*Patinopecten yessoensis*) and oysters (*Crassostrea gigas*), where global methylation levels increase during the oogenesis and spermatogenesis stages, coinciding with high *DNMT3* expression (Zhang *et al.* 2018; Li *et al.* 2019). For example, in zebrafish, promoter methylation of reproductive genes such as *esr1* (estrogen receptor alpha) and *amh* (anti-Müllerian hormone) is associated with sex-biased gene expression in both gonads and liver, while epigenetic regulators like *dnmt1*, *dnmt3*, and *hdac1* are more highly expressed in ovaries than in testes, indicating a sex-specific methylation landscape governing gonadal function (Laing *et al.* 2018; Li *et al.* 2019).

Sexual maturity prediction has been advanced using conserved CpG sites to develop predictive models for reproductive timing. By using tissue-derived DNA methylation signal, Heydenrych *et al.* (2024) demonstrated that CpG density in promoter regions can accurately predict sexual maturity age. The correlation coefficients of their models were 0.81 in females and 0.76 in males, supporting the notion that methylation mediates the regulation of reproductive processes. This approach is particularly beneficial for endangered species or those that are difficult to observe longitudinally in the wild.

Gestation and fertility have also been linked to DNA methylation through the development of epigenetic clocks. Li *et al*. (2024b) found that gestational duration correlated with methylation levels in placental tissues, achieving a correlation coefficient = 0.96 in model predictions, emphasizing the potential of epigenetic clocks in reproductive ecology. Sperm quality and fertility are influenced by methylation as well: in striped bass, MBD-Seq

identified 171 DMRs distinguishing high- and low-fertility sperm, enriched in genes such as WDR3/UTP12 and GPCRs (Woods III *et al.* 2018), while in common carp (*Cyprinus carpio*), *in vitro* sperm aging is associated with temporal methylation changes that correlate with declines in motility and fertilization rate, peaking at 24 hours post-stripping and declining by 96 hours (Cheng *et al.* 2021). The observed stage-specific methylation patterns during gametogenesis and regulation of hormonal and metabolic pathways suggest it may contribute to coordinating reproductive cycles and energy allocation.

Epi-eDNA has also shown potential in reproductive ecology. Hirayama *et al.* (2024) demonstrated that spikes of unmethylated DNA, a characteristic of germ cell genomes, appeared in water samples during the peak spawning periods of fish. Since complementary unmethylated rDNA accounts for most rDNA repeats in unfertilized eggs, their release leads to a transient but detectable signature in eDNA, compared to the high methylation rate detected in somatic cells. Thus, the sharp shift in the eDNA methylation signals served as an indicator of spawning events. Importantly, methylation patterns confined to gonadal tissues are unlikely to be detected in eDNA unless germ cells are released; therefore, the primary application of epi-eDNA is the detection of spawning events.

Health status and stress responses

Epigenetic modifications are responsive to physiological stress and disease, making epi-eDNA a potential indicator of ecosystem health, as it can reflect the current biological state of organisms and the environmental pressures affecting entire communities. Balard *et al.* (2024) reviewed the potential of DNA methylation signals to assess health conditions in wildlife populations. If specific methylation changes are linked to exposure to pollutants or pathogens, their detection in eDNA could indicate an early warning signal of ecosystem stress or disease outbreaks. Although still in a conceptual phase, studies have shown that stressors such as chemical pollutants or infections can induce reproducible methylation changes (Cavalieri & Spinelli 2017).

In marine mammals, anthropogenic stressors such as underwater noise and tourism pressure are of growing concern. These impacts are complicated to quantify. While cortisol levels provide a measurement of short-term stress, DNA methylation analysis offers a non-invasive method for assessing medium- to long-term stress, which could be valuable for cetacean conservation (Crossman *et al.* 2021). Recent studies have demonstrated this potential. For example, killer whales (*Orcinus orca*) were found to have distinct methylation patterns in stress-response genes between populations exposed to different levels of human activity, suggesting cumulative stress can be detected epigenetically (Crossman *et al.* 2021). In bottlenose dolphins (*Tursiops* spp.), DNA methylation-based epigenetic clocks not only estimated age with high accuracy but also showed that individuals with accelerated epigenetic aging—i.e., a higher DNA methylation age than expected—tended to have lower

health scores that are significantly related to the survival probabilities (Barratclough *et al.* 2024). These findings highlight the utility of DNA methylation as an indicator of age and health impacts, enabling more precise and long-term monitoring of cetacean populations under human pressure.

Epi-eDNA thus provides a powerful tool for monitoring organismal response to environmental stress, particularly in scenarios where direct biological sampling in the field is not feasible. For example, temperature and salinity can induce methylation changes in promoter regions of specific genes in crustaceans (Guo *et al.* 2025) and fish (Blondeau-Bidet *et al.* 2023). Promoters with varying CpG densities are associated with distinct gene functions, and alterations in their methylation states—detected through epi-eDNA—can be used to infer whether organisms are experiencing stress and to assess the level of stress. However, there are differences in methylation patterns and functions among different species (Klughammer *et al.* 2023), and the broad applicability of epi-eDNA still needs further verification.

In line with this concept, Hishikawa *et al.* (2024) reported that accelerated DNA methylation age and increased DNA damage in urinary shedding cells are significantly correlated with current renal function and predict future renal deterioration. Their pilot study on patients with chronic kidney disease (CKD) showed that epigenetic age acceleration—measured using Hannum's and PhenoAge clocks—was strongly associated with both reduced estimated glomerular filtration rate (eGFR) and its rate of decline. Moreover, DNA double-strand breaks detected in urine-derived renal cells, particularly those associated with proximal tubule markers, were closely linked to biological aging and functional decline. These clinical findings, though focused on human kidney health, highlight the potential of methylation-based signatures in excreted DNA to noninvasively monitor systemic stress, offering a compelling analogy for health surveillance in wildlife via environmental DNA.

As sensitivity improves and new methylation signals are validated, it may become feasible to routinely monitor population health and environmental stressors through analysis of epi-eDNA in water or soil samples.

Conclusions and perspectives

In summary, research on DNA methylation in environmental DNA (epi-eDNA) has just emerged in recent years. From its conceptual inception in 2019, when researchers first proposed that methylated cytosines in eDNA could be detected in environmental samples (Sigsgaard *et al.* 2019), several empirical studies in recent years support the idea (Hanski *et al.* 2024; Hirayama *et al.* 2024; Ruiz *et al.* 2025; Yagi *et al.* 2024; Zhao *et al.* 2023). Recent studies confirm that epi-eDNA may stably capture functional information at both the individual and population levels. These findings position epi-eDNA as a promising method for ecological inference. Looking forward, continued advances in sequencing technologies

and methylation detection methods will likely unlock the full potential of epigenetic information embedded in eDNA. As methodological sensitivity improves, epi-eDNA is poised to play a transformative role in ecological monitoring, resource management, and biodiversity conservation (Balard *et al.* 2024).

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Author contributions

CL and MU conceived and planned this review; CL and MU wrote the first draft; CL summarized and visualized the literature review; CL, FX, IH, GY, and MU reviewed and edited the manuscript; MU performed the project administration and supervision.

Competing interests

The authors declared no competing interests.