Trends in
Ecology & Evolution

CellPress

Opinion

Environmental RNA: A Revolution in Ecological Resolution?

Matthew C. Yates,[1,*] Alison M. Derry,[1] and Melania E. Cristescu[2]

Current advancements in environmental RNA (eRNA) exploit its relatively fast turnover rate relative to environmental DNA (eDNA) to assess 'metabolically active' or temporally/spatially recent community diversity. However, this focus significantly underutilizes the trove of potential ecological information encrypted in eRNA. Here, we argue for pushing beyond current species-level eDNA detection capabilities by using eRNA to detect any organisms with unique eRNA profiles, potentially including different life-history stages, sexes, or even specific phenotypes within a species. We also discuss the future of eRNA as a means of assessing the physiological status of organisms and the ecological health of populations and communities, reflecting ecosystem-level conditions. We posit that eRNA has the potential to significantly improve the resolution of organism detection, biological monitoring, and biomonitoring applications in ecology.

Highlights

eDNA has revolutionized species-detection and biomonitoring applications.

Recent research has shown that eRNA can be extracted from aquatic environments.

Current eRNA applications exploit its rapid degradation to quantify temporally recent or metabolically active communities.

Next-generation sequencing (NGS) provides unprecedented ability to quantify the transcriptome.

The application of NGS to study eRNA could significantly improve the resolution of organism detection and biological monitoring.

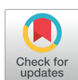
Harnessing eRNA to Improve Organism Detection and Biological Monitoring

The analysis of **eDNA** (see Glossary) in aquatic ecosystems has become widespread in ecology [1]. Conversely, the study of **eRNA** is still in its infancy [2]. Under laboratory conditions, RNA is a less stable molecule compared with DNA. As a result, it has been assumed that RNA in environmental media degrades rapidly and cannot be detected in biologically meaningful quantities. However, recent laboratory experiments demonstrated that environmental mRNA can be readily extracted and detected [3–5], decay rate constants for eRNA vary [5] but can be surprisingly similar to those of eDNA [4], environmental mRNA can be detected in water samples for up to 13–72 h after production [4,5], and eRNA concentrations can even be higher than corresponding eDNA markers [5]. In nature, eRNA can also be detected at high concentrations in close proximity to study organisms [6]. The potential high turnover rate of eRNA has led to a renewed interest in using it to detect organisms or make ecological inferences in 'real time' because it could reflect the presence or absence of species with higher spatial and temporal resolution compared with eDNA [2]. While dead organisms often shed copious amounts of DNA, RNA is expected to be produced mainly by physiologically active organisms. Thus, eRNA might better reflect metabolically active assemblages of organisms [2,7,8] by partially circumventing the high rates of false positives that can occur with eDNA-based methods. However, we propose here that a focus on detecting 'living assemblages' or recent species occupation could underutilize its potential. We argue that eRNA could offer substantially increased detection resolution beyond traditional eDNA surveys and novel diagnostic applications.

eRNA, unlike eDNA, reflects the transcriptomic profile of progenitor organisms. This difference is critically important, because organisms that share (near) identical genetic profiles may exhibit substantial differences in their **transcriptome**, depending on their developmental state, physiological state, genetic background, or phenotype [9–14]. RNA has a crucial role in **gene expression**, either as a functional gene product [**noncoding RNA** (ncRNA)] or as **mRNA**, which translates genes into proteins [15]. The **transcription** rate of genes can vary substantially, with some genes strongly

[1]Département des Sciences Biologiques, Université du Québec à Montréal, 141 Avenue Président-Kennedy, Montréal, QC, H2X 1Y4, Canada
[2]Department of Biology, McGill University, 1205 Dr Penfield Avenue, Montreal, QC, H3A 1B1, Canada

*Correspondence:
matthew.yates@outlook.com
(M.C. Yates).



© 2021 Elsevier Ltd. All rights reserved.

upregulated or downregulated depending on the ecological niche, physiological state, life-history stage, or phenotype of an organism [9,10,16,17]. Since RNA sequences are only produced for genes that are actively transcribed within the tissues of an organism, a similar transcriptional signature could be reflected in the eRNA that an organism produces, a signature that could be detectable for some time [4,5]. We believe that this represents a potentially important, but currently overlooked, aspect of eRNA. Specifically, eRNA could enable several novel applications (Figure 1), including utilizing eRNA to move targeted high-specificity assay (Box 1) monitoring capabilities beyond the species level to detect any organisms that produce a unique or strongly upregulated eRNA transcript. This could potentially be exploited to both detect different conspecific forms (e.g., life-history stages, sexes, phenotypes, etc.) and/or monitor the physiological state of organisms in nature. Additionally, environmental metatranscriptomics (Box 1) could be used to monitor the physiological responses of communities and track ecosystem-level conditions. We also discuss further research needed to study eRNA production and degradation, as well as technical challenges with its collection and preservation.

Utilizing High-Specificity eRNA Assays to Increase Biological Monitoring Resolution

Pushing Detection Limits beyond the Species Level

Current **environmental nucleic acid** (eNA) applications largely focus on DNA. Conspecific organisms typically have similar genetic profiles and, as a result, the current resolution of eDNA methodologies limits its application to species detection or limited population-level genetic inferences that exploit genetic differences among populations (e.g., haplotype frequencies) [18]. For example, it is not possible to differentiate eDNA sourced from juveniles and adults from

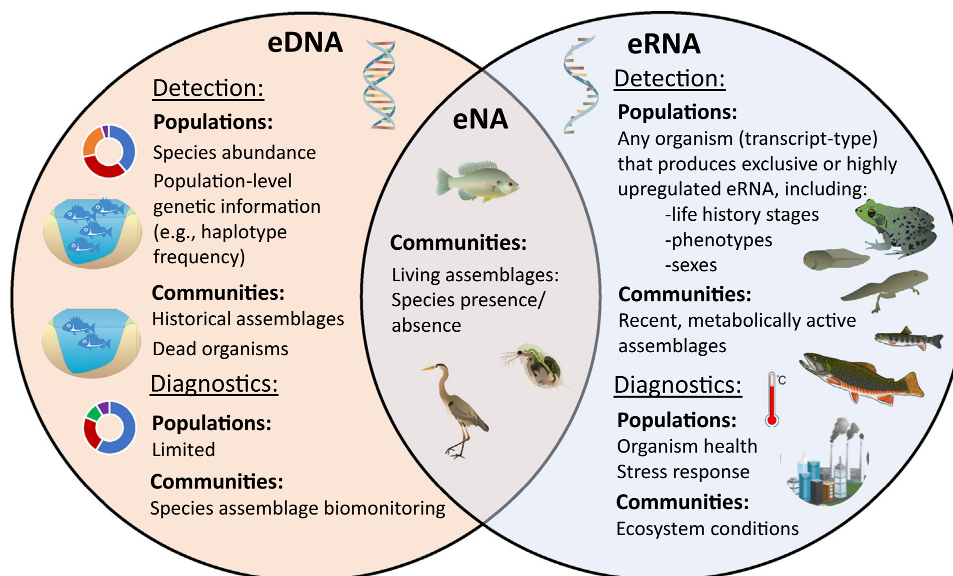


Figure 1. Environmental RNA (eRNA) Has the Potential to Significantly Improve Population and Community Biological Monitoring Applications by Moving beyond Environmental DNA (eDNA) Detection of Species Presence, Abundance, and Diversity towards Enhanced Detection and Diagnostics of Population and Community Characteristics. Illustrations by Tracy Saxby, Emily Nastase, Kim Kraeer, and Jane Thomas; Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/imagelibrary/). Illustrations of Brook trout by Shannon Clarke.

Glossary

Environmental DNA (eDNA): DNA released by the organisms in an environmental medium (e.g., soil, water, or air). Defined here in a narrow sense as extra-organismal (e.g., as free form, cellular, absorbed to mineral or organic compounds, etc.).

Environmental nucleic acid (eNA): any extra-organismal nucleic acid (DNA or RNA) found in an environmental medium.

Environmental RNA (eRNA): extra-organismal RNA found in an environmental medium (e.g., as free form, in vesicles, or cellular), as opposed to RNA derived from organisms collected in bulk samples.

Environmental (meta) transcriptomics: traditionally refers to sequencing microbial RNA from a bulk sample to generate community gene expression profiles, often using metatranscriptomics approaches. The term is used herein to refer to gene expression profiling based on eRNA, which could apply to both eukaryotes (extra-organismal RNA) and prokaryotes (organismal RNA), and spanning all trophic levels.

Environmental transcript-type: any form of an organism that produces unique or heavily upregulated eRNA sequences, potentially including (but not limited to) specific sexes, phenotypes, developmental stages, genotypes, and/or populations.

Gene expression: process of converting DNA sequence information into a functional gene product (typically proteins or noncoding RNA).

Metabarcoding: DNA-based identification method involving the extraction of multiple species from a mass collection of specimens (bulk samples) or from samples of eDNA. Also known as amplicon sequencing, this method can be applied effectively to quantify microbial communities, meio-, and megafauna.

Metatranscriptomics: see 'Environmental Transcriptomics'

mRNA: RNA sequences transcribed from protein-coding DNA that are converted into amino acid-based proteins by ribosomes. The sequence of mRNA codons determines the composition and order of amino acids assembled by the ribosome, which ultimately determines the structure of the protein that is synthesized.

the same species/population from DNA sequence data alone. While conspecific organisms share near-identical genetic profiles, their transcriptome can vary substantially depending on many factors, including (but not limited to) genetic background, developmental stage, sex, and phenotype [13,14,16,19,20]. We speculate that transcriptional differences between different forms of conspecific organisms (e.g., life-history stages, phenotypes, etc.) may be reflected in the eRNA they produce. Ultimately, any organismal form (which we refer to as an '**environmental transcript-type**') that exclusively produces or heavily upregulates a specific eRNA transcript relative to other conspecifics could potentially be detected from environmental samples by using a targeted high-specificity assay to detect and/or quantify such eRNA sequences (Box 1). Assays targeting such genes would need to be species specific (i.e., would not amplify similar genes in related species) and genes would need to exhibit differential expression among conspecifics. The identification of suitable candidate genes is currently limited by the lack of transcriptomic data among all but the most well-studied species [3]. Additionally, eRNA expression for biomarkers that are 'upregulated' (as opposed to exclusively produced) by environmental transcript-types would likely need to be 'normalized' relative to other 'housekeeping' genes and/or organism abundance (Box 2). Nevertheless, eRNA represents a promising alternative to current monitoring approaches to quantify or detect different forms of conspecifics organisms, which are currently limited to physical specimen collection methods that can be invasive, costly, and ineffective in difficult-to-sample contexts.

Environmental RNA to Monitor the Physiological State of Organisms

Environmental conditions can commonly induce physiological responses in organisms. For example, heat-shock proteins are commonly produced when organisms are exposed to thermal stress [21,22]. Previous research has exploited such responses to develop RNA biomarkers that can indicate exposure to specific environmental conditions. Organisms exposed to different **stressors**, for example, can exhibit substantial differences in transcriptomic profiles [17,23,24]. Transcriptional responses to a variety of environmental conditions and/or stressors (e.g., thermal stress, pH, salinity, dissolved oxygen, toxic pollutants, bacterial/viral infection, etc.) have been identified across a range of aquatic organisms, including corals [25,26], macroinvertebrates [27], zooplankton [28], molluscs [29], and vertebrates [11,12,17,23,30,31].

Such transcriptional responses may also be reflected in the eRNA produced by organisms, including markers that are exclusively or heavily upregulated under specific conditions. Current methods that utilize transcriptomic responses to infer the physiological, toxicological, or immune status of organisms either require animal sacrifice or costly, labor-intensive, and invasive tissue collections (e.g., gill or fin clips) [12,17,23,30,32]. The capacity to evaluate the physiological status of organisms from eRNA would significantly improve monitoring efforts to evaluate organism health in natural environments [2,3].

Notably, eDNA is often applied for the detection of rare species at low densities [33], and this framework shapes sampling considerations and perceptions of 'false-negative' risks. However, environmental RNA may be most useful under conditions of moderate-to-high densities, when organisms are known to occur a priori but their physiological status is unknown. Therefore, it is important not to impose sampling concerns associated with rare species detection on potential eRNA applications without prior empirical justification; 'steady-state' concentrations of eRNA from common species at high densities may well be higher than concentrations of eDNA from rare species. One study found that the likelihood of eRNA detection in nature increased with eDNA copy number, with >400 copies of eDNA in a sample predicting eRNA detection [6]; this threshold is regularly exceeded when monitoring eDNA concentrations of common species at high densities [34–36].

Noncoding RNA (ncRNA): RNA sequences transcribed from DNA regions that do not encode protein, including (but not limited to) long ncRNAs (lncRNA), miRNA, tRNA, and rRNA.
Quantitative PCR (qPCR): molecular technique used to detect and/or quantify the concentration of a target DNA sequence by monitoring the PCR amplification of the target sequence in real time using nonspecific intercalating dyes or fluorescently labeled oligonucleotide probes.
RNA-seq: methods that use next-generation sequencing technology to generate RNA sequence data in the form of reads that, using bioinformatics approaches, can be assembled to provide genome-wide transcriptome data with levels of gene expression based on transcript read number.
RT-qPCR: molecular technique similar to qPCR except it targets a specific RNA sequence. RNA is first converted to cDNA before qPCR (two-step RT-qPCR) or during the initial step of the qPCR reaction (one-step RT-qPCR), after which cDNA is quantified using qPCR.
Stressor: environmental variable exceeding a normal range of values tolerated by organisms, resulting in adverse impacts on individual organisms, species, communities, and/or ecosystems. Exposure can often induce plastic gene expression.
Transcription: process of converting DNA sequence information into RNA.
Transcriptome: total RNA transcripts (including coding and noncoding) in an organism.

Box 1. Molecular Approaches to Detect and Quantify Environmental RNA

High-Specificity Targeted Approaches

qPCR is a sensitive technique that can detect or quantify the copy number of a specific DNA sequence (Table I). It is commonly used in species-specific eDNA detection applications because it can provide absolute copy number quantification and detect target sequences at low concentrations [55]. The inclusion of hydrolysis probes (e.g., TAQMAN™) can significantly improve assay specificity. **RT-qPCR** is an analogous technique for RNA, and can be used for eRNA applications [4]. Care should be taken to design assays that avoid co-amplification of RNA sequences from the same gene in related species [56] or related genes within the same species [3,9]. Due to the current lack of transcriptomic libraries available for all but the most commonly studied organisms, assay specificity should, at a minimum, be tested on closely related species that might co-occur in nature. Digital droplet PCR (ddPCR) may be particularly effective (relative to RT-qPCR) at detecting RNA from environmental samples due to its improved performance when target sequence copy number is low [57], higher precision [58], and resilience to inhibitors [59], although it requires substantially higher infrastructure costs.

Metabarcoding (e.g., Universal Primer) Approaches

Metabarcoding approaches seek to assess community diversity from eDNA samples by matching PCR-amplified eDNA sequences in environmental samples to standardized databases of species-specific sequences [60]. Metabarcoding can also be used on eRNA sequences for inventory-based applications [7,61]. Primers used in PCR-based metabarcoding are 'universal' (i.e., will amplify sequences from a variety of taxa) and are particularly useful for assaying biodiversity due to their broad community-level resolution [62], although metabarcoding can often be less sensitive than species-specific approaches [63], cannot provide absolute copy number quantification, requires extensive gene libraries [60], and can be costly. However, metabarcoding approaches could be particularly useful for quantifying RNA transcripts from genes conserved across species.

Metatranscriptomics Approaches

Next-generation sequencing technology (e.g., RNA-seq) has enabled the development of methods that sequence any RNA present in environmental samples (referred to as 'environmental metatranscriptomics'). Metatranscriptomics has traditionally been applied to study microbial ecological communities [46]. Whether macro-organism (e.g., eukaryotic) RNA can be detected using metatranscriptomic approaches remains unknown, and is a major area of future investigation. Metatranscriptomics can effectively quantify microbial RNA because metabolically active microorganisms are collected as a component of environmental samples. However, macro-organismal eRNA is extra-organismal and thus expected to degrade rapidly and form a smaller proportion of eRNA present in samples.

Table I. Characteristics of Different Technical Sequencing Approaches to Quantify RNA in Environmental Samples

Approach	High specificity	Broad community resolution	Low limit of detection	Absolute copy number quantification	Low cost	High precision	Resilient to inhibition	Multi-species libraries	Targeted (sequence specific)
qPCR	✓	–	~	✓	✓	~	–	–	✓
ddPCR	✓	–	✓	✓	–	✓	✓	–	✓
Metabarcoding	~	✓	–	–	–	–	–	✓	✓
Metatranscriptomics	–	✓	–	–	–	–	NA	✓	–

Approaches to Developing High-Specificity eRNA Assays

Recent laboratory experiments validated the use of targeted approaches [e.g., quantitative (q) PCR/droplet digital (dd)PCR] to reliably detect and quantify specific eRNA genes [4,5]. We propose two potential approaches to identifying and developing high-specificity eRNA biomarkers, each with distinct advantages and challenges. A promising potential approach is a 'library-first' approach, which would seek to construct libraries of potential eRNA biomarkers from tissues or whole organisms that would be subsequently validated on eRNA samples. Initial experimental approaches could focus on quantifying whole-organism or tissue-specific transcriptomes in organisms experimentally exposed to a gradient of environmental conditions or in different potential environmental transcript-types, after which tissue RNA that is uniquely expressed or heavily up/downregulated in relevant treatments can be identified (e.g., [17,20]). Several high-specificity assays could then be developed targeting potential biomarkers, which can then be validated on eRNA samples collected from experimental replicates or natural environments [3]. Controlled experimental approaches have the distinct advantage that tissue and eRNA samples can be simultaneously collected from experimental replicates to infer causal linkages.

A rich literature already exists for some species documenting transcriptional responses to environmental conditions (e.g., stressors) or potential environmental transcript-types. Sequence-specific

Box 2. Normalizing Upregulated eRNA Biomarkers Using Housekeeping Genes or eDNA

The Role of 'Housekeeping' Genes in eRNA Assays

The highest resolution eRNA biomarkers would be exclusively expressed by specific environmental transcript-types or under specific environmental conditions. However, many relevant potential genes are upregulated instead. Such genes can still be expressed at low relative levels in the absence of a stressor [11] or by other potential environmental transcript-types (e.g., developmental stages) [13,38]. RNA expression can also vary considerably among individuals or based on variability in the quantity of starting material [14,64]. A common strategy in gene expression studies is to normalize RNA expression levels to standard 'house-keeping' genes expressed at stable levels under most environmental conditions [64]. Upregulated genes detected in eRNA could still be normalized against housekeeping genes, assuming that housekeeping genes can be reliably detected in eRNA in consistent quantities. However, the question of what constitutes a stable 'eRNA' gene is unknown. Little research has examined how different forms of RNA are expressed in eRNA or degrade in the environment (but see [5]).

Normalizing eRNA Biomarkers for Organism Abundance Using eDNA

An additional problem also facing the use of 'upregulated' eRNA biomarkers is that changes in the abundance of organisms, including those with low levels of expression, could account for observed changes in the concentration of upregulated eRNA sequences in natural environments, obscuring their reliability as biomarkers for specific environmental conditions or environmental transcript-types. Therefore, 'upregulated' eRNA biomarkers must also be normalized to an index of organism abundance. Environmental DNA concentration can exhibit strong correlations with species abundance in nature [34,65,66]. Similar relationships might be found with eRNA; however, given the significant variability in individual-level transcription levels coupled with the instability of RNA, we predict that correlations between eRNA and abundance will likely be positive but moderate in strength relative to eDNA, even among putative 'housekeeping' genes. Alternatively, 'upregulated' eRNA markers could potentially be normalized to organism abundance by comparing eRNA concentrations to the concentration of an eDNA marker from the same organism. However, careful consideration must be given to how environmental conditions affect the relationship between eDNA production, organism abundance, and eRNA biomarkers; for example, temperature can increase eDNA production rates in poikilothermic organisms [67]. Confident assessments of population health from eRNA would also require extensive spatial sampling efforts to capture environmental heterogeneity in an ecosystem and simultaneously sample eRNA from several individuals to reduce the impact of interindividual variation.

eRNA biomarkers developed in such studies could be applied directly to eRNA samples from relevant natural environments, or novel assays could be designed based on developed transcriptomic libraries. Model species may be particularly well suited for initial studies; for example, transcriptomic responses to stressors and in different life-history stages (e.g., juvenile-to-adult metamorphosis) have been well studied for organisms such as *Daphnia* [10,24] or *Xenopus laevis* (African clawed frog) [9]. Well-studied socioeconomically important fish species also represent promising systems, with transcriptional biomarkers previously identified for immune challenges [12], temperature [11,17], pollutants [23], maturation [13,19,37], and metamorphosis [20,38,39]. Pacific salmonids, in particular, might represent ideal initial study species: they can occur at high densities during spawning (increasing the likelihood of eRNA recovery in nature), are exposed to several environmental challenges (e.g., high temperatures, oxygen deprivation, immune challenges, etc.), and undergo physiological changes associated with life-history phenology.

The primary challenge associated with a library-first approach is the unknown extent to which potential biomarker sequences developed from whole-organism or tissue-specific transcriptomes will be reflected in the eRNA produced by organisms (Box 3). Developing potential eRNA markers from tissue transcriptomes therefore has risks; substantial effort could be invested in creating novel assays or studying previously designed biomarkers that target tissue-specific RNA sequences that were never a detectable component of the eRNA produced by study organisms. Nevertheless, we believe that a library-first approach represents a promising method to develop eRNA assays because focusing efforts on tissue types likely to produce eRNA (e.g., digestive, epidermal, respiratory, and reproductive tissues) could mitigate some of this risk [3] (Box 3).

Alternatively, 'eRNA-first' approaches would attempt to first quantify the eRNA organisms produce, and then develop targeted assays based on the genes identified. This approach avoids uncertainty around the relative representation of various tissue transcriptomes in eRNA.

Box 3. Understanding eRNA Production, Degradation, and Improving eRNA Sample Preservation

Improving Our Understanding of eRNA Production

Transcription rates for genes can vary significantly in different tissues [13]. The extent to which the transcription rates of different genes within the tissues of an organism and transcriptional changes in response to environmental conditions are reflected in the eRNA it produces is unknown; the composition and sources of eRNA produced by an organism represent priority areas for future research. Does the eRNA produced by an organism resemble a homogenized mixture of RNA expressed in a variety of differentiated cells, or is it biased towards a small number of specific cell types/organs? Given that the two main sources of the production of eDNA are thought to be cells shed from the surface of an organism and the excretion of metabolic wastes and reproductive fluids [68], the composition of eRNA is likely biased toward genes commonly expressed in the digestive system of an organism, in tissues in direct contact with environmental mediums (e.g., epidermis and respiratory systems), and in reproductive systems (particularly organisms with external fertilization) [3].

Quantifying eRNA Degradation

Research on eRNA degradation is limited. In particular, there is a need to study relative degradation rates of different forms of RNA [e.g., mRNA, long ncRNA (lncRNA), and rRNA]; thus far, a single study found faster degradation rates for nuclear eRNA relative to mitochondrial eRNA, and variable rates for rRNA and mRNA [5]. Intracellular stability of RNA transcripts varies substantially, with half-lives ranging from <2 h to >16 h [69]. Several mechanisms can affect the intracellular stability of RNA, including secondary structure, RNA-binding proteins, and active cellular degradation [69,70]. Candidate RNA genes for biomonitoring applications should ideally exhibit relatively high stability in natural environments, but do not necessarily need to be limited to protein-coding mRNA. ncRNAs have been implicated in sex-, developmental-, or tissue-specific regulatory roles [15]. For example, lncRNA can have significant roles in up- or downregulating gene expression and in cell signaling [15,71].

Improving eRNA Sample Preservation

Additional research on methods to improve the quantity and quality of RNA collected and preserved in environmental samples is crucial. Important avenues of research include investigating the effect of immediate filtration [72], the addition of chemical reducing agents to filter storage buffers [73,74], and the immediate freezing of samples [4,6]. Nevertheless, it is clear that RNA exists in environmental mediums [2] and can successfully be detected [6]; methodologies to preserve it need only be refined.

High-volume eRNA samples would need to be collected from experimental replicates with high densities of organisms exposed to a gradient of stressor treatments or environmental transcript-types and then sequenced using next generation high-throughput sequencing (**RNA-seq**) [40]. Environmental samples are nonselective and often include organismal RNA or eRNA from microorganisms (e.g., bacteria, algae, microeukaryotes, etc.) that cohabit or are commensal, symbiotic, or parasitic with study organisms. To exclude sequences from nontarget organisms, eRNA sequence data could be mapped to the genome and/or transcriptome of the target species using previously assembled reference genomes/transcriptomes or, alternatively, tissue transcriptome data from organisms in the same experiment (e.g., *de novo* assemblies). Bioinformatics approaches [40] can then be used to identify RNA sequences that are consistently upregulated or exclusively detected in the eRNA of relevant organisms.

However, significant research is necessary before eRNA-first approaches can be implemented. Broad sequencing approaches (e.g., shotgun/RAD sequencing, genotyping-by-sequencing, etc.) are typically inefficient methods to quantify eukaryote eNAs in environmental samples because most nucleic acids in environmental samples are likely to be (i) low concentration; (ii) low-quality/partially degraded; and (iii) potentially sourced mainly from prokaryotes. For example, eDNA from live prokaryotes collected in an environmental sample can 'drown-out' eDNA from macroeukaryotes during 'shotgun' sequencing [41]; similar processes are likely to affect relative eRNA recovery. However, various methods that deplete bacterial rRNA [42–44] or sequester eukaryotic mRNA (e.g., poly-A hybridization) [45] could improve its relative recovery [46]. Similarly, macro-organisms in nature typically occur at low relative densities compared with conditions achievable in laboratories, and environmental samples from natural ecosystems contain nucleic acids produced by a diverse assemblage of eukaryotes [41]. Collecting large-volume eRNA samples

from experimental replicates containing solely the focal species at high densities with conditions unfavorable to microbial growth could improve eukaryotic eRNA recovery by increasing its relative concentration and ensuring the sole presence of macroeukaryotic eRNA from focal species. Results from initial aquaria experiments are promising; several studies estimated high concentrations of specific eRNA genes for invertebrate species [4,5], implying a substantial quantity of total species-specific eRNA in the aquaria.

Using Environmental Transcriptomics to Monitor Ecosystem-Level Conditions

An intriguing prospect represents the use of bulk mRNA released by organisms into their environment to infer both the taxonomic composition and gene-expression profile of a complex community of macro-organisms. Traditionally, metagenomics has been used to reconstruct the taxonomic composition of communities [47]. Such information is based on DNA and does not reflect community responses to specific environmental conditions. Alternatively, **metatranscriptomics** uses RNA and, as a result, can provide a comprehensive profile of the genes transcribed to support the structure and function of organisms at the time of sampling. Complex community profiling based on gene transcript abundance is sensitive to environmental conditions [48,49] and (although currently costly) could be integrated into monitoring programs to provide information on ecosystem health.

Metatranscriptomic approaches have traditionally been applied to study microbial communities, where mRNA is extracted from bulk samples containing live bacteria; only a handful of studies have been conducted on eukaryotes [50]. RNA profiles analyzed in these studies are likely dominated by organismal RNA sourced from live micro-organisms, with potentially small amounts of extra-organismal RNA. However, a similar approach could be extrapolated to macroeukaryotes by extracting extra-organismal mRNA from environmental samples in the absence of progenitor organisms. **Environmental transcriptomics** represents a non-invasive means to study the functional activity of a community of organisms that does not require prior knowledge of community species composition or specific genes expressed under particular conditions. From such functional data, active metabolic pathways can be identified and linked to particular environmental conditions or stressors.

Many technical difficulties associated with the application of metatranscriptomics to eRNA could be envisioned. Eukaryotic organisms have larger and more complex genomes compared with prokaryotes, requiring higher sequence coverage. Total microbial RNA samples often comprise only ~1–5% mRNA reads, with most being rRNAs and tRNAs [51]. mRNA reads likely represent only a small fraction of the total eRNA, with macro-organismal mRNA probably representing an even smaller fraction. However, several techniques can improve the relative recovery of eukaryotic mRNA [46] (see earlier). Analytical approaches also pose significant challenges. An important challenge when using transcriptomics as a monitoring tool is having a baseline for comparison, a 'control' under optimal environmental conditions. However, in the absence of such experimental controls, changes in transcript levels (RNA) could be compared with changes in gene abundance (template DNA). A mismatch between RNA and DNA copies could reflect differential expression, or functional changes. Metatranscriptomics is yet to be validated as a tool for biomonitoring because its cost, coupled with the amount and complexity of data produced, is intimidating [50,52]. Nevertheless, environmental transcriptomics based on eRNA represents a promising approach for assessing community responses to environmental conditions across trophic levels [2].

Concluding Remarks

eDNA has revolutionized organism detection [53] and has the potential to advance biomonitoring [54], but its resolution is limited to species-level detection or, at present, limited population-level genetic inferences [18]. eRNA has the potential to push beyond these capabilities and improve

Outstanding Questions

Can we reliably quantify eRNA produced by organisms?

Is the composition of eRNA a homogenous mixture of RNA sources from a variety of tissues and species, or is its composition biased towards a few tissue types and few species?

To what extent does the eRNA produced by ecologically and physiologically discrete individuals (i.e., potential environmental transcript-types) differ?

What is the relative contribution of different life-history stages, phenotypes, sexes, and so on, to the eRNA found in an environment?

Are transcriptional responses to stressors reflected in eRNA produced by organisms exposed to them?

Can transcriptional responses in the eRNA of common organisms be reliably associated with specific environmental conditions?

What types of genes and RNA (e.g., mRNA, ncRNA, etc.) tend to be represented in eRNA?

What are the relative concentrations of different forms of RNA in the environment?

What is the relative concentration of eRNA compared with eDNA in natural ecosystems?

How does the concentration of eRNA in an environment vary with organism abundance?

What is the turnover rate of different forms of RNA (e.g., mRNA, lncRNA, rRNA, miRNA, etc.) in various environments?

How does RNA quantity/quality vary with sample volume?

What is the effect of immediate filtration on RNA quantity/quality?

How rapidly does RNA degrade after collection/filtration?

Can the preservation of RNA in environmental samples be enhanced using chemical reducing agents (e.g., 2-mercaptoethanol) that deactivate RNases in storage buffers?

the resolution of identification beyond the species level to detect any organisms with a unique eRNA signature. Similarly, transcriptional responses reflected in eRNA could be used to non-invasively monitor the health of organisms or communities and track ecosystem-level conditions. Environmental RNA could substantially increase the level of resolution provided by eNA assays, with significant implications and broad applications for the study of ecology, evolution, and wildlife management. Empirical studies investigating eRNA for organism detection, biological monitoring, and biomonitoring applications are crucial to unlock this potential. However, further research is necessary to understand the ecology of eRNA and improve the efficiency of its preservation and capture (see Outstanding Questions).

What effect does immediate freezing (e.g., with liquid nitrogen) have on the preservation of RNA in environmental samples?

Acknowledgments
M.C.Y. was supported by a Fonds de Recherche du Québec - Nature et Technologies (FRQNT) post-doctoral fellowship and a FRQNT team research operating grant to A.M.D. and M.E.C.

Declaration of Interests
None declared by authors.

References
1. Cristescu, M.E. and Hebert, P.D.N. (2018) Uses and misuses of environmental DNA in biodiversity science and conservation. *Annu. Rev. Ecol. Evol. Syst.* 49, 209–230
2. Cristescu, M.E. (2019) Can environmental RNA revolutionize biodiversity science? *Trends Ecol. Evol.* 34, 694–697
3. Tsuri, K. *et al.* (2021) Messenger RNA typing of environmental RNA (eRNA): a case study on zebrafish tank water with perspectives for the future development of eRNA analysis on aquatic vertebrates. *Environ. DNA* 3, 14–21
4. Wood, S.A. *et al.* (2019) Release and degradation of environmental DNA and RNA in a marine system. *Sci. Total Environ.* 704, 135314
5. Marshall, N.T. *et al.* (2021) Environmental (e)RNA advances the reliability of eDNA by predicting its age. *Sci. Rep.* 11, 1–11
6. Von Ammon, U. *et al.* (2019) Linking environmental DNA and RNA for improved detection of the marine invasive fanworm *Sabella spallanzanii*. *Front. Mar. Sci.* 6, 621
7. Pochon, X. *et al.* (2017) Wanted dead or alive? Using metabarcoding of environmental DNA and RNA to distinguish living assemblages for biosecurity applications. *PLoS ONE* 12, e0187636
8. Laroche, O. *et al.* (2018) A cross-taxa study using environmental DNA/RNA metabarcoding to measure biological impacts of offshore oil and gas drilling and production operations. *Mar. Pollut. Bull.* 127, 97–107
9. Watanabe, Y. *et al.* (2002) Metamorphosis-dependent transcriptional regulation of xak-c, a novel *Xenopus* type I keratin gene. *Dev. Dyn.* 225, 561–570
10. Spanier, K.I. *et al.* (2017) Conserved transcription factors steer growth-related genomic programs in daphnia. *Genome Biol. Evol.* 9, 1821–1842
11. Akbarzadeh, A. *et al.* (2018) Developing specific molecular biomarkers for thermal stress in salmonids. *BMC Genomics* 19, 749
12. Miller, K.M. *et al.* (2017) Molecular indices of viral disease development in wild migrating salmon. *Conserv. Physiol.* 5, cox036
13. Ciani, E. *et al.* (2020) General and comparative endocrinology Gnrh receptor gnrhr2bb α is expressed exclusively in lhb-expressing cells in Atlantic salmon male parr. *Gen. Comp. Endocrinol.* 285, 113293
14. Debes, P.V. *et al.* (2012) Differences in transcription levels among wild, domesticated, and hybrid Atlantic salmon (*Salmo salar*) from two environments. *Mol. Ecol.* 21, 2574–2587
15. Rinn, J.L. and Chang, H.Y. (2012) Genome regulation by long noncoding RNAs. *Annu. Rev. Biochem.* 81, 145–156
16. Prado-lima, M. and Val, A.L. (2015) Differentially expressed genes in the pituitary of the Amazonian fish *Arapaima gigas*. *Int. J. Fish. Aquac.* 7, 132–141
17. Houde, A.L.S. *et al.* (2019) Salmonid gene expression biomarkers indicative of physiological responses to changes in salinity and temperature, but not dissolved oxygen. *J. Exp. Biol.* 222, jeb198036
18. Sigsgaard, E. *et al.* (2020) Population-level inferences from environmental DNA – current status and future perspectives. *Evol. Appl.* 13, 245–262
19. Skaftnesmo, K.O. *et al.* (2017) Integrative testis transcriptome analysis reveals differentially expressed miRNAs and their mRNA targets during early puberty in Atlantic salmon. *BMC Genomics* 18, 801
20. Houde, A.L.S. *et al.* (2019) Discovery and validation of candidate smoltification gene expression biomarkers across multiple species and ecotypes of Pacific salmonids. *Conserv. Physiol.* 7, coz051
21. Jeffries, K.M. *et al.* (2012) Consequences of high temperatures and premature mortality on the transcriptome and blood physiology of wild adult sockeye salmon (*Oncorhynchus nerka*). *Ecol. Evol.* 2, 1747–1764
22. Chen, B. *et al.* (2018) Evolution of heat-shock protein expression underlying adaptive responses to environmental stress. *Mol. Ecol.* 27, 3040–3054
23. Baillon, L. *et al.* (2016) Detecting the exposure to Cd and PCBs by means of a non-invasive transcriptomic approach in laboratory and wild contaminated European eels (*Anguilla anguilla*). *Environ. Sci. Pollut. Res.* 23, 5431–5441
24. Ravindran, S.P. *et al.* (2019) Daphnia stressor database: taking advantage of a decade of *Daphnia* '-omics' data for gene annotation. *Sci. Rep.* 9, 11135
25. Parkinson, J.E. *et al.* (2018) Extensive transcriptional variation poses a challenge to thermal stress biomarker development for endangered corals. *Mol. Ecol.* 27, 1103–1119
26. Hou, J. *et al.* (2018) RNA-seq reveals extensive transcriptional response to heat stress in the stony coral *Galaxea fascicularis*. *Front. Genet.* 9, 37
27. Drozdova, P. *et al.* (2019) Comparison between transcriptomic responses to short-term stress exposures of a common Holarctic and endemic Lake Baikal amphipods. *BMC Genomics* 20, 712
28. Orsini, L. *et al.* (2018) Early transcriptional response pathways in *Daphnia magna* are coordinated in networks of crustacean specific genes. *Mol. Ecol.* 27, 886–897
29. Nie, H. *et al.* (2017) High throughput sequencing of RNA transcriptomes in *Ruditapes philippinarum* identifies genes involved in osmotic stress response. *Sci. Rep.* 7, 4953
30. Jeffries, K.M. *et al.* (2014) Transcriptomic responses to high water temperature in two species of Pacific salmon. *Evol. Appl.* 7, 286–300

31. Oomen, R.A. and Hutchings, J.A. (2017) Transcriptomic responses to environmental change in fishes: insights from RNA sequencing. *Facets* 2, 610–641
32. Baillon, L. *et al.* (2015) Transcriptome profile analysis reveals specific signatures of pollutants in Atlantic eels. *Ecotoxicology* 24, 71–84
33. Jerde, C.L. (2019) Can we manage fisheries with the inherent uncertainty from eDNA? *J. Fish Biol.* 98, 341–353
34. Yates, M.C. *et al.* (2021) The relationship between eDNA particle concentration and organism abundance in nature is strengthened by allometric scaling. *Mol. Ecol.* 3 (2), 343–352
35. Wilcox, T.M. *et al.* (2016) Understanding environmental DNA detection probabilities: a case study using a stream-dwelling char *Salvelinus fontinalis*. *Biol. Conserv.* 194, 209–216
36. Doi, H. *et al.* (2017) Environmental DNA analysis for estimating the abundance and biomass of stream fish. *Freshw. Biol.* 62, 30–39
37. Schulz, R.W. *et al.* (2019) Entry into puberty is reflected in changes in hormone production but not in testicular receptor expression in Atlantic salmon (*Salmo salar*). *Reprod. Biol. Endocrinol.* 17, 48
38. Robertson, L.S. and McCormick, S.D. (2012) Transcriptional profiling of the parr-smolt transformation in Atlantic salmon. *Comp. Biochem. Physiol. - Part D Genomics Proteomics* 7, 351–360
39. Karlsen, C. *et al.* (2018) Atlantic salmon skin barrier functions gradually enhance after seawater transfer. *Sci. Rep.* 8, 9510
40. Wang, Z. *et al.* (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* 10, 57–63
41. Stat, M. *et al.* (2017) Ecosystem biomonitoring with eDNA: Metabarcoding across the tree of life in a tropical marine environment. *Sci. Rep.* 7, 12240
42. Culviner, P.H. *et al.* (2020) A simple, cost-effective, and robust method for rRNA depletion in RNA-sequencing studies. *MBio* 11, e00010-20
43. Broman, E. *et al.* (2020) High throughput shotgun sequencing of eRNA reveals taxonomic and derived functional shifts across a benthic productivity gradient. *Mol. Ecol.* Published online July 24, 2020. https://doi.org/10.1111/mec.15561
44. He, S. *et al.* (2010) Validation of two ribosomal RNA removal methods for microbial metatranscriptomics. *Nat. Methods* 7, 807–812
45. Damon, C. *et al.* (2012) Metatranscriptomics reveals the diversity of genes expressed by eukaryotes in forest soils. *PLoS ONE* 7, e28967
46. Shakya, M. *et al.* (2019) Advances and challenges in metatranscriptomic analysis. *Front. Genet.* 10, 904
47. Sunagawa, S. *et al.* (2015) Structure and function of the global ocean microbiome. *Science* 348, 1261359
48. Shi, Y. *et al.* (2011) Integrated metatranscriptomic and metagenomic analyses of stratified microbial assemblages in the open ocean. *ISME J.* 5, 999–1013
49. Moran, M.A. *et al.* (2013) Sizing up metatranscriptomics. *ISME J.* 7, 237–243
50. Vorobev, A. *et al.* (2020) Transcriptome reconstruction and functional analysis of eukaryotic marine plankton communities via high-throughput metagenomics and metatranscriptomics. *Genome Res.* 30, 647–659
51. Peano, C. *et al.* (2013) An efficient rRNA removal method for RNA sequencing in GC-rich bacteria. *Microb. Inform. Exp.* 3, 1
52. Birrer, S.C. *et al.* (2019) Using meta-omics of contaminated sediments to monitor changes in pathways relevant to climate regulation. *Environ. Microbiol.* 21, 389–401
53. Goldberg, C.S. *et al.* (2015) Moving environmental DNA methods from concept to practice for monitoring aquatic macroorganisms. *Biol. Conserv.* 183, 1–3
54. Pawlowski, J. *et al.* (2018) The future of biotic indices in the ecogenomic era: integrating (e)DNA metabarcoding in biological assessment of aquatic ecosystems. *Sci. Total Environ.* 637–638, 1295–1310
55. Klymus, K.E. *et al.* (2020) Reporting the limits of detection and quantification for environmental DNA assays. *Environ. DNA* 2, 271–282
56. Wilcox, T.M. *et al.* (2013) Robust detection of rare species using environmental DNA: the importance of primer specificity. *PLoS ONE* 8, e59520
57. Taylor, S.C. *et al.* (2017) Droplet digital PCR versus qPCR for gene expression analysis with low abundant targets: from variable nonsense to publication quality data. *Sci. Rep.* 7, 2409
58. Doi, H. *et al.* (2015) Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. *PLoS ONE* 10, e0122763
59. Capo, E. *et al.* (2020) Droplet digital PCR applied to environmental DNA, a promising method to estimate fish population abundance from humic-rich aquatic ecosystems. *Environ. DNA* Published online June 24, 2020. https://doi.org/10.1002/edn3.115
60. Taberlet, P. *et al.* (2012) Towards next-generation biodiversity assessment using DNA metabarcoding. *Mol. Ecol.* 33, 2045–2050
61. Zaiko, A. *et al.* (2018) Advantages and limitations of environmental DNA/RNA tools for marine biosecurity: management and surveillance of non-indigenous species. *Front. Mar. Sci.* 5, 322
62. Valentini, A. *et al.* (2016) Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Mol. Ecol.* 25, 929–942
63. Harper, L.R. *et al.* (2018) Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (*Triturus cristatus*). *Ecol. Evol.* 8, 6330–6341
64. Bustin, S.A. (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* 25, 169–193
65. Spear, M.J. *et al.* (2020) Application of eDNA as a tool for assessing fish population abundance. *Environ. DNA* 3, 83–91
66. Yates, M.C. *et al.* (2019) Meta-analysis supports further refinement of eDNA for monitoring aquatic species-specific abundance in nature. *Environ. DNA* 1, 5–13
67. Jo, T. *et al.* (2019) Effect of water temperature and fish biomass on environmental DNA shedding, degradation, and size distribution. *Ecol. Evol.* 9, 1135–1146
68. Stewart, K.A. (2019) Understanding the effects of biotic and abiotic factors on sources of aquatic environmental DNA. *Biodivers. Conserv.* 28, 983–1001
69. Clark, M.B. *et al.* (2012) Genome-wide analysis of long noncoding RNA stability. *Genome Res.* 22, 885–898
70. Yoon, J. *et al.* (2015) Long noncoding RNA turnover. *Biochimie* 117, 15–21
71. Geisler, S. and Coller, J. (2013) RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. *Nat. Rev. Mol. Cell Biol.* 14, 699–712
72. Hinlo, R. *et al.* (2017) Methods to maximise recovery of environmental DNA from water samples. *PLoS ONE* 12, e0179251
73. Van der Poel-van de Luytgaarde, S.C.P.A.M. *et al.* (2013) Addition of b-mercaptoethanol is a prerequisite for high-quality RNA isolation using QIAsymphony technology as demonstrated by detection of molecular aberrations in hematologic malignancies. *Genet. Test. Mol. Biomarkers* 17, 475–480
74. Mommaerts, K. *et al.* (2015) Replacing β-mercaptoethanol in RNA extractions. *Anal. Biochem.* 479, 51–53