Title:

The promise of environmental RNA research beyond mRNA

Running Title:

eRNA beyond mRNA

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Abstract:

Environmental RNA (eRNA) studies have primarily focused on species detection and community composition through metabarcoding or metatranscriptomics, and on gene expression through messenger RNA (mRNA) abundance analysis. While valuable, this focus overlooks the broader functional roles of other RNA types in cellular metabolism. Beyond mRNA, non-coding RNAs as well as structural RNAs play critical roles in gene regulation during stress responses, development, and adaptation. Additionally, RNA processes like RNA methylation or alternative splicing also respond to similar environmental or developmental signals. When applied to eRNA research, these additional RNA types and RNA processes hold significant potential as powerful, non-invasive tools for monitoring physiological state of entire species communities. In this roadmap, we present underexplored RNA types and processes relevant for eRNA research, outlining their functions and the challenges of integrating them into the field. Expanding eRNA research to include more diverse aspects of RNA biology will require improved experimental techniques for sensitive and reliable detection and quantification of specific RNAs in eRNA samples, along with enhanced tools for taxonomic and functional annotation and the expansion of genetic reference databases. Where species-level resolution is not possible, functional inferences could be drawn at higher taxonomic levels. Expanding the scope of eRNA studies to encompass more diverse RNA types and RNA processes will provide additional insights into species communities' state, their adaptation potential and responses to stressors in a non-invasive way.

Keywords:

Environmental RNA, Ecosystem monitoring, RNA methylation, rRNA, miRNA, non-coding RNA

1. Introduction

The analysis of environmental RNA (eRNA) - RNA molecules released into the environment and collected from environmental samples - is emerging as a powerful, non-invasive tool for obtaining valuable ecological and physiological insights for target organisms or even entire communities (Cristescu, 2019; M. C. Yates, Furlan, Thalinger, Yamanaka, & Bernatchez, 2023; Matthew C. Yates, Derry, & Cristescu, 2021). eRNA metabarcoding and metatranscriptomics have been shown to complement or even outperform eDNA approaches in community assessments due to higher turnover time, more localized signals and stronger indication of living organisms (Giroux, Reichman, Langknecht, Burgess, & Ho, 2022; Greco et al., 2022; Macher et al., 2024). But the true power of eRNA analysis could lie in its ability to provide information on physiological condition or functional characteristics of organisms. Since the RNA molecules in an eRNA sample are actively transcribed molecules in the bodies of organisms that have been released into the environment, their abundance can reflect real-time gene expression changes linked to physiological states, such as stress responses, metabolism, immune activity, or development, offering insight into the condition of organisms at the time of release. However, to date, the focus of this functional research has been on mRNA abundance, studying gene expression to infer ecosystem dynamics. While this approach has been informative, it only scratches the surface of RNA's diverse roles in environmental systems. Expanding the focus to other aspects of RNA dynamics can reveal critical insights into stress responses, microbial interactions, and ecosystem stability, thereby broadening the scope of eRNA research. A cell's RNA metabolism is a complex interplay involving different types of RNAs participating in various chemical processes, where they can act as targets, catalysts, or both. These processes govern a wide range of metabolic pathways, from protein synthesis to immune responses, providing valuable insights into multiple aspects of the ecology and evolution of species, communities, and ecosystems (Figure 1). In this opinion paper, we present several aspects of RNA biology beyond gene expression that could be relevant for eRNA research. Hereby we discuss 1) RNA types aside from mRNA and 2) RNA processes that regulate, modify and process RNA. The RNA types discussed include regulatory RNAs, such as miRNAs and siRNAs, which play key roles in stress responses, microbial interactions, and long-term adaptation, as well as structural, translational, and catalytic RNAs, such as rRNAs and tRNAs, which are essential for metabolic changes and adaptation (see details below). The discussed RNA processes include RNA modification like methylation, pseudouridylation and polyadenylation are pivotal for RNA stability or translation.

Despite their potential to address broader ecological and evolutionary questions, many of these approaches remain underexplored, partly driven by technical challenges that need to be overcome, as discussed in more detail below. These challenges include detecting low-abundance RNAs, lack of genetic

reference data or low genetic resolution, interpreting transient modifications, and analyzing complex RNA structures. To overcome these, advanced laboratory techniques, such as specialized RNA-seq methods and mass spectrometry, must be adapted for use in environmental samples. Computational tools also need improvement to support better annotation, splicing analysis, and detection of RNA modifications. This review outlines the potential directions for eRNA research beyond mRNA studies (Figure 2). By exploring diverse RNA types and processes, researchers can deepen their understanding of stress adaptation, microbial interactions, and ecosystem resilience. These advancements will provide valuable insights into ecological stability and help address pressing environmental challenges such as climate change and biodiversity loss.

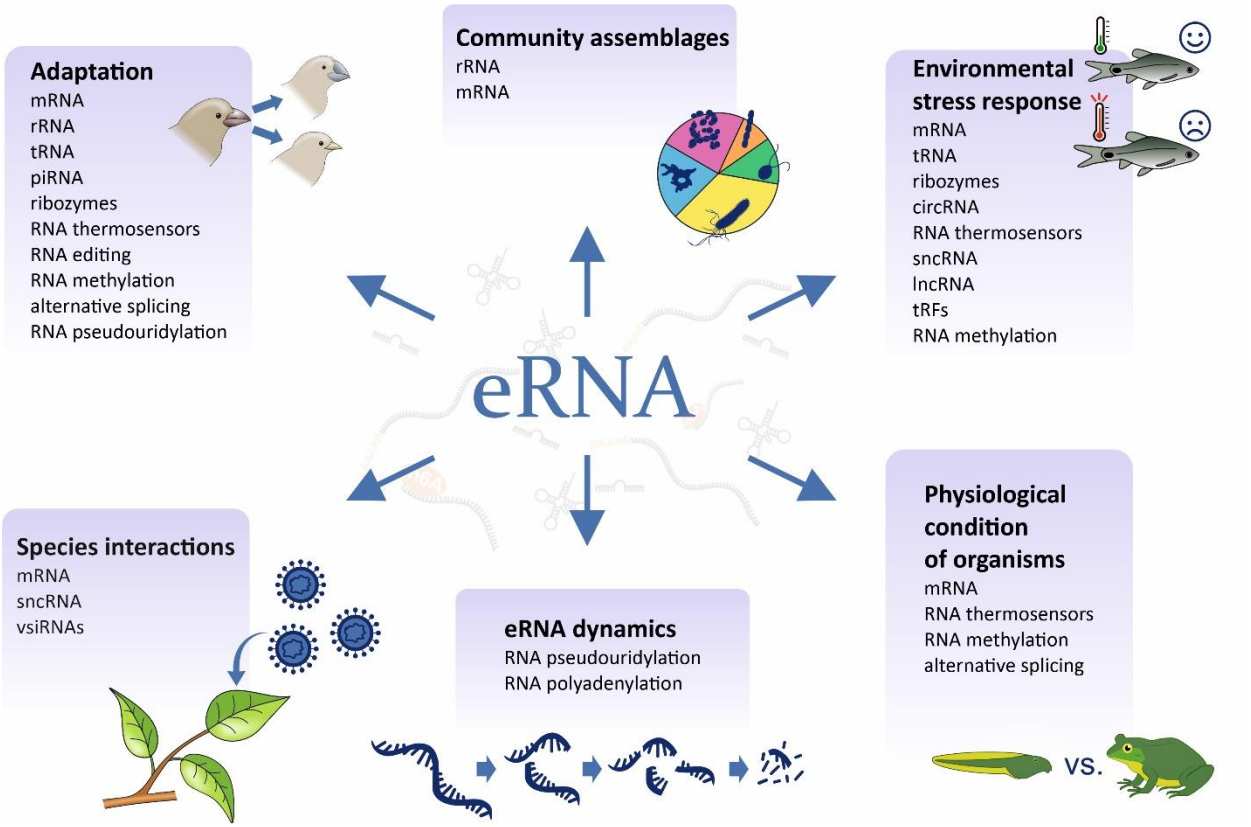

Figure 1: Research topics that could be addressed with the RNA types and RNA processes discussed in this manuscript. Boxes list the individual topics in bold with the RNA types and RNA process being informative on the respective topic given below. Note that individual RNA types and processes can be listed more than once.

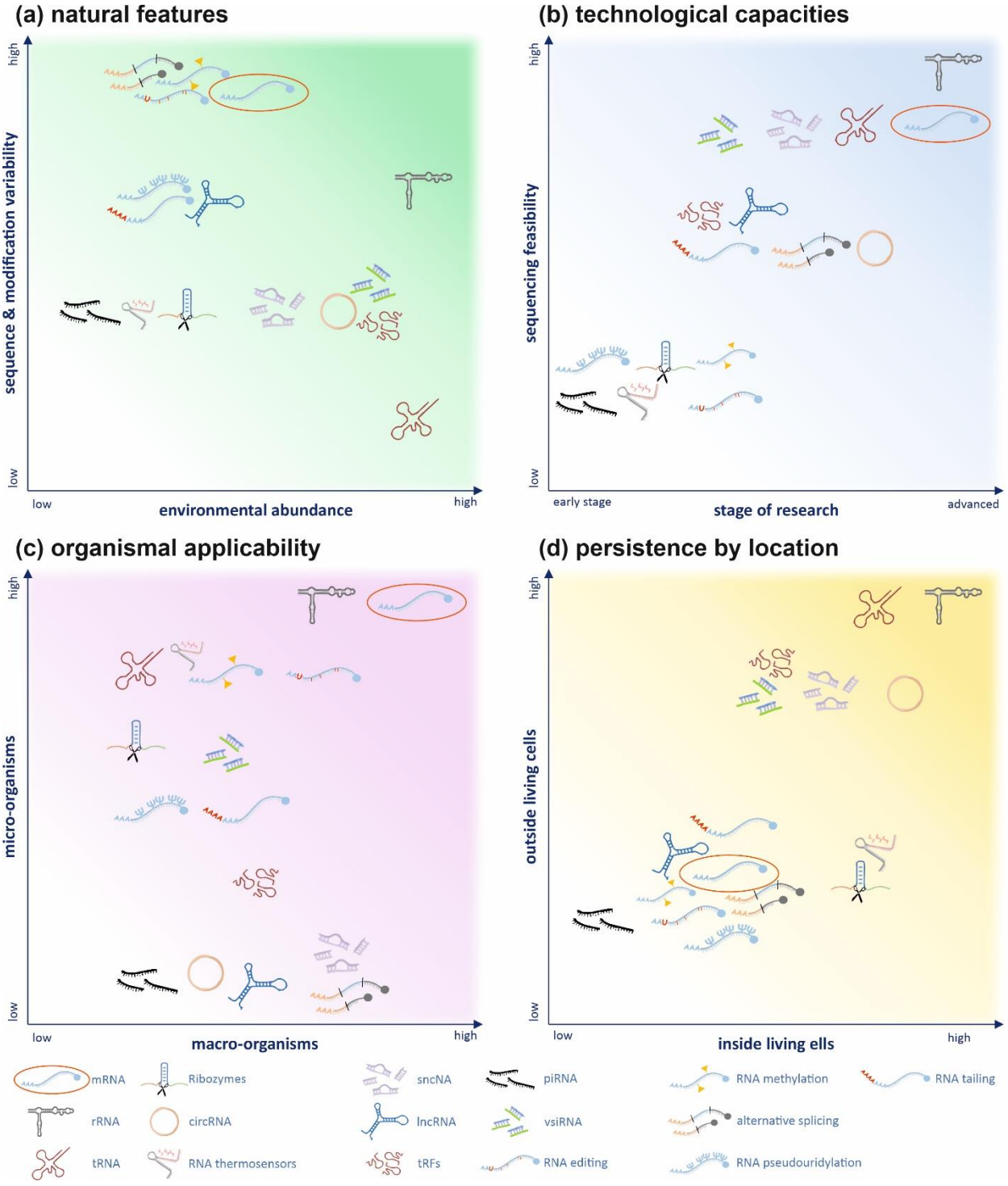

Figure 2: Natural features (a), technological capacities (b), organismal applicability (c), and environmental persistence (d) of RNA types/processes influencing their current suitability for eRNA research. Environmental abundance refers to the abundance of the RNA type in environmental samples, while sequence & modification variability is determined by molecule length and genetic diversity. Sequencing feasibility illustrates the need for deep sequencing or specialized library preparation, sequencing methods, or other techniques. Stage of research indicates the extent of existing studies on the respective RNA type or process. Organismal applicability refers to the relevance of RNA

types/processes for studying micro- or macro-organisms. Persistence by location illustrates the potential lifespan of RNA types/processes inside or outside organisms. Please note that the placement of RNA types/processes in this figure is purely relative to mRNA and simplified and based on general knowledge of their characteristics (e.g., size, variation, abundance, sequencing feasibility under laboratory conditions, and existing literature) and require additional experimental verification. Potential species-specific and environment-dependent variations are not considered.

2. RNA types

A summary of the RNA types discussed in this section is provided in Table 1, including their functions, significance for environmental studies, and associated limitations.

2.1. Ribosomal RNA (rRNA)

Ribosomal RNA (rRNA) forms the core of the ribosome's structure and plays a critical role in the translation of genetic information from DNA into proteins. rRNA serves as already now a reliable marker for assessing microbial community composition and activity from eRNA samples through eRNA metabarcoding, due to its stability and ubiquity across all domains of life (An, Mun, & Kim, 2023; Blazewicz, Barnard, Daly, & Firestone, 2013; Giroux, Reichman, Langknecht, Burgess, & Ho, 2023; Miyata et al., 2021; Yan, Zou, Zhu, Hozzein, & Quan, 2017). Its high sequence conservation makes rRNA a preferred target for taxonomic identification and phylogenetic studies, providing insights into species diversity and ecosystem function (Adamo, Voyron, Chialva, Marmeisse, & Girlanda, 2020; Xue et al., 2024) and it's high abundance and stability make it a dependable biomarker even in degraded samples like eRNA samples (Cholet, Ijaz, & Smith, 2019; Kagzi et al., 2023). However, the high conservation of rRNA can also limit its resolution for differentiating closely related species. Furthermore, functional rRNA studies primarily reflect the potential for variation in protein translation of an organism, rather than the true functional variability in gene expression. In future eRNA studies, rRNA can continue to be applied to monitor active species communities, assess ecosystem health at macro- and micro-organismal levels, and explore the dynamics of communities in response to environmental changes (Gao et al., 2023) but expanding its application to include rRNA modifications could uncover novel adaptations to stress or nutrient availability. For example, in microbes, rRNA sequence variants have been linked to environmental stress such as heat shock or nutrient limitation (Leppek & Barna, 2019) and modifications of rRNA, such as methylation (see also below) have been proposed as indicators of various environmental stressors (heat, cold, starvation, oxygen stress) across the tree of life (Baldridge & Contreras, 2014). Therefore, detecting specific sequence variants or identifying rRNA methylation patterns from eRNA samples could indicate micro- or microbial

species communities under such stressors. Furthermore, variation in rRNA gene copy number has been shown to reflect differing evolutionary strategies in responding to environmental changes (Klappenbach, Dunbar, & Schmidt, 2000), suggesting that analyzing copy number variation in environmental samples could reveal a species community's capacity to respond to environmental changes.

2.2. Transfer RNA (tRNA)

Transfer RNA (tRNA) is essential for protein synthesis, serving as the adaptor molecule between mRNA and amino acids during translation. Hereby, tRNA can be modified affecting its function in proteins synthesis (Björk & Hagervall, 2014; Schauss, Kundu, Fingerhut, & Elsaesser, 2021). Different tRNA levels and modifications can serve as indicators of cellular metabolic states, stress responses, and adaptive strategies (Chan, Lin, Mak, & Lowe, 2021; Hoffmann et al., 2024; Huber, Leonardi, Dedon, & Begley, 2019; Murakami, Fujishima, Tomita, & Kanai, 2012; Schauss et al., 2021; Schwartz et al., 2018). tRNA is more stable than mRNA (Prossliner, Agrawal, Heidemann, Sørensen, & Svenningsen, 2023) suggesting longer persistence times and more robust detection probabilities in eRNA samples. However, its high abundance might obscure subtle changes, and the functional significance of many tRNA modifications remains poorly understood (Abe et al., 2014) highlighting the need for further in vivo research. Furthermore, its high conservation may complicate taxonomic annotation in mixed-origin samples like eRNA samples. However, while this limits the ability to attribute inferred functions to specific species, the conserved nature of tRNA may still allow for functional inferences at broader taxonomic levels, such as family, class, or even kingdom, within an ecosystem or habitat. Potential applications of tRNA analysis from environmental samples include the discovery of tRNA modifications in extreme environments, which may reveal molecular mechanisms of adaptation (Hoffmann et al., 2024; Sharma, Zhang, Ehrenkaufer, & Singh, 2023). For instance, the detection of specific tRNA modifications, such as methylation or thiolation at defined nucleotide positions, could serve as indicators of community-level stress responses to temperature extremes (Lorenz, Lünse, & Mörl, 2017; Rashad et al., 2024). Additionally, examining codon bias (i.e., the difference in occurrence of different tRNAs encoding for the same amino acid) in eukaryotic communities could reveal active stress responses, as codon bias can reflect the regulation of critical response proteins to environmental stressors (Endres, Dedon, & Begley, 2015).

2.3. Ribozymes

Ribozymes are RNA molecules with enzymatic activity, that can catalyze chemical reactions without any protein involved (Papastavrou, Horning, & Joyce, 2024). They play key roles in various aspects of protein synthesis, like for RNA splicing (the removal of introns from RNA during protein synthesis) or translation (Wilson & Lilley, 2021) (Wilson & Lilley, 2021). At an organismal level, ribozymes contribute to key processes like nutrient cycling and energy metabolism, especially in microbial communities (Peri, Gibard, Shults, Crossin, & Hayden, 2022; Popović, Fliss, & Ditzler, 2015) and they have been suggested as powerful markers for adaptation (Belfort, 2017; Hayden & Wagner, 2012). However, ribozymes are often rare and challenging to detect, requiring targeted sequencing and biochemical validation. The activity of ribozymes can be influenced by physical (temperature, light) or chemical (ligands) signals (Frommer, Appel, & Müller, 2015), therefore the study of ribozymes structure and activity from environmental samples could reveal community or species responses or adaptation to certain environmental settings.

2.4. Circular RNA (circRNA)

Circular RNAs (circRNAs) are RNA molecules that lack free ends, making them exceptionally stable compared to linear RNAs (Xuebing Chen, Xu, Lin, & Zhu, 2024; Verwilt & Vromman, 2024). They act as regulators during gene expression through various processes (Ebbesen, Hansen, & Kjems, 2017) and have been suggested to be an underexplored layer of gene regulation (Lasda & Parker, 2016). Their stability makes them a particularly promising target for eRNA-based studies. However, the functional implications of genetic variation in circRNA is still poorly understood at an organismal or environmental level, and their detection may require sophisticated bioinformatics tools (Drula, Braicu, & Neagoe, 2024). In livestock, expression profiles of circular RNAs have been linked to development and growth (Meng et al., 2020; S. H. A. Raza et al., 2022). Hence, quantifying expression levels of these molecules in environmental samples could be used to infer physiological conditions of vertebrate communities. Furthermore, circDNA expression is known to be highly dynamic across different developmental stages (Dang et al., 2016; Fischer & Leung, 2017; Szabo et al., 2015), suggesting that expression levels of circRNA in eRNA samples could be used to trace the developmental status of vertebrates within ecosystems. Expression levels of circRNAs have also been shown to vary in response to thermal and hypoxic stress in Echinodermata (Huo et al., 2021) and various plants (Kalwan et al., 2023; X. Yang et al., 2020), further supporting their potential as indicators of stress in both animal and plant communities.

2.5. RNA thermosensors

RNA thermosensors are RNA molecules that sense and respond to temperature changes by altering their conformation (Mandin & Johansson, 2020; Somero, 2018). These changes regulate gene expression, typically at the level of translation, enabling organisms to adapt to thermal fluctuations. Commonly studied in bacteria, RNA thermosensors are known to control heat-shock protein expression (Abduljalil, 2018; Mandin & Johansson, 2020; Steinmann & Dersch, 2013). RNA thermosensors offer a unique mechanism to study real-time thermal adaptation, providing insights into how organisms sense and respond to temperature changes, something conventional mRNA studies cannot address (Catalan-Moreno et al., 2021; A. Raza, Siddique, & Hu, 2024; Roncarati, Vannini, & Scarlato, 2024; Steinmann & Dersch, 2013). However, identifying thermosensors requires resource-intensive techniques like SHAPE-seq and is often limited to bacterial systems (Meyer, Carlson, & Lucks, 2017), leaving their broader ecological roles underexplored. In future eRNA studies, sequencing or structural analysis of thermosensors could be used to detect ecosystem thermal stress such as temperature mediated pathogen outbreaks (Loh, Righetti, Eichner, Twittenhoff, & Narberhaus, 2018; Noll et al., 2019; Twittenhoff, Heroven, Mühlen, Dersch, & Narberhaus, 2020). Hereby, ongoing technological advances may soon make it feasible to describe the secondary and tertiary structures of these molecules directly from environmental samples. This could allow researchers to determine whether resident species communities have reached critical thermal thresholds triggering physiological stress responses.

2.6. Small non-coding RNAs

Small non-coding RNAs (sncRNAs), such as microRNAs (miRNAs), piwi-interacting RNAs (piRNAs) or RNA virus-derived siRNAs are short RNA molecules that do not encode proteins but play critical roles in regulating gene expression at the post-transcriptional level by binding to target mRNAs, leading to their degradation or translational inhibition. On an organismal level, sncRNAs have been shown to mediate stress responses, regulate microbial community interactions, and other inter-organismal communication (Asgari, 2017; Xuemei Chen & Rechavi, 2021; Chi et al., 2023; Nawaz & Wang, 2022; Weiberg, Wang, Bellinger, & Jin, 2014). The regulation of gene expression by small non-coding RNAs has been suggested to provide understanding of RNA-mediated regulation in ecosystems which are not captured in mRNA abundance studies (Weiberg, Bellinger, & Jin, 2015). However, their short sequences and diverse targets pose challenges for their analysis in complex, mixed-origin environmental samples, such as taxonomic classifications or functional interpretation (Gelsinger et al., 2020; Heintz-Buschart et al., 2018; Lott, Voigt, Lambrecht, Hess, & Steglich, 2020). Nevertheless, in future eRNA studies, sncRNAs could be instrumental

in elucidating interspecies communication across kingdoms, host pathogen interactions and community-level stress signaling (Xuemei Chen & Rechavi, 2021; Chi et al., 2023; Gualtieri, Leonetti, & Macovei, 2020; Sarshar, Scribano, Palamara, Ambrosi, & Masotti, 2022). For instance, pathogen-derived sncRNAs have been shown to modulate gene expression in their plant hosts (Regmi, Penton, Anderson, & Gupta, 2022; M. Wang, Weiberg, Dellota, Yamane, & Jin, 2017). When applied to eRNA samples, abundance profiling of specific sncRNAs could help identify active pathogen-host interactions within a given species community.

miRNAs: MicroRNAs (miRNAs), typically less than 25 nucleotides long, play crucial roles in gene regulation during development, immune responses and adaptation to stress (Carrington & Ambros, 2003; Leung & Sharp, 2010). Their small sizes may complicate taxonomic annotation but their high abundance relative to other sncRNAs (Lu et al., 2005; C. Zhang, 2008), their stability due to frequent packaging in extracellular vesicles (J. Zhang et al., 2015) and our comparatively advanced understanding of their functions makes them promising targets for eRNA research nevertheless - particularly when inferences are drawn at higher taxonomic levels. In animals, miRNA have been implicated in regulating metabolically depressed state, such as during mammalian hibernation, in freeze-tolerant insects, anoxia-tolerant amphibians and reptiles and, in bivalves exposed to toxins (Abo-Al-Ela & Faggio, 2021; Biggar & Storey, 2018). As such, the analysis of miRNA expression profiles could identify vertebrate species communities currently experiencing environmental conditions that triggered extensive physiological reactions. This could, in turn, reveal community-level adaptation strategies and coping mechanisms in the assessed communities.

piRNAs: Piwi-interacting RNAs (piRNAs) are animal specific, sncRNAs that protect genomes from transposable elements (Ozata, Gainetdinov, Zoch, O'Carroll, & Zamore, 2018) mostly in cells of the germ line (like eggs or sperm). Transposable elements are mobile fractions of the genome that insert themselves into new places and can originate from an organism's own genome but also from parasitic infections such as viral infections. Since they are particularly crucial for protecting the germline of transposable elements, they can have a profound effect onto the fitness of the next generation (Belicard, Jareosettasin, & Sarkies, 2018; Ozata et al., 2018). In environmental contexts, they could play roles in stress adaptation and genome stability (Casier, Boivin, Carré, & Teysset, 2019; Casier, Delmarre, et al., 2019; Ter Horst, Nigg, Dekker, Falk, & Pfeiffer, 2019). Therefore, the assessment of presence and diversity of piRNA in a species community could not only elucidate the current community's ability to fight off viral infections or to cope with environmental stressors, but it could also infer the next generation's ability to adapt to and cope with these stressors.

vsiRNAs: Small interfering RNAs derived from viral RNA (vsiRNA) are small RNA molecules that are produced by a host organism in response to infection by an RNA virus and are important for the host's immune defense by silencing viral genes in plants, invertebrates, and some vertebrates (Li, Weng, Shih, & Brewer, 2016; Llave, 2010). vsiRNAs therefore highlight active viral infections and host responses. However, similar to other sncRNAs, they are challenging to detect and to assign to individual species due to their small size and low abundance. As vsiRNAs are used as defense systems against viral infections viral RNA, quantifying these RNA types in communities would enable researchers to identify and track viral infections of entire communities and ecosystems.

2.7. Long non-coding RNAs

Long non-coding RNAs (lncRNAs) is a large and highly diverse group of RNA molecules longer than 200 nucleotides that do not encode proteins but are involved in diverse cellular processes, such as transcriptional regulation, chromatin remodeling, and gene expression control (Toomer, Gan, & Sztuba-Solinska, 2020). Similar to sncRNAs, lncRNAs are implicated to be involved in stress responses, organismal adaptation, and microbial-host interactions (Fahad, Tariq, Muhammad, & Wu, 2024; Ou et al., 2022; Qu et al., 2019; H. Yang et al., 2023; X. F. Zhao et al., 2021). But unlike sncRNAs, lncRNAs operate already at the transcriptional level and on chromatin levels, meaning they are affecting larger areas of the genome and influence gene expression more globally and fundamentally than sncRNAs. However, their functions are less conserved across species, requiring a better understanding of their functions on a species-by-species basis first. Currently, our understanding of lncRNAs' roles in environmental stress response is more advanced in plants, while knowledge in animals or microorganisms is still very limited (Nejat & Mantri, 2018; Song & Zhang, 2017; H. Yang et al., 2023). Once a better understanding of their function has been established, the sequence and/or expression analysis of lncRNAs from environmental samples could further help to elucidate stress responses of species communities.

2.8. tRNA fragments (tRFs)

tRNA fragments (tRFs) are small RNA molecules derived from the cleavage of tRNAs (Tosar & Cayota, 2020). These fragments have regulatory roles, such as repressing translation, modulating stress responses, and influencing inter-organismal signaling (Lalande, Merret, Salinas-Giegé, & Drouard, 2020; Margis, Eguiluz, Guzman, Rodrigues, & Dias-Oliveira, 2023; Z. Sun et al., 2022). However, they are poorly characterized in environmental contexts, and detecting them requires specialized sequencing techniques (Kimura, Srisuknimit, & Waldor, 2020; Lakshmanan et al., 2021; Molla-Herman et al., 2020). Furthermore,

their small size complicates genomic and taxonomic annotation, particularly from mixed-origin samples such as eRNA samples, and distinction between in-cell cleavage and degradation in the environment might be hard to achieve. However, certain stress conditions can induce cleavage of tRNAs into tRFs (Saikia et al., 2012), the analysis of accumulation of tRFs in relation to their progenitor tRNAs in environmental samples could indicate the presence of environmental stressors onto species assemblies.

3. RNA processes

A summary of the RNA processes discussed in this section is provided in Table 2, including associated functions, significance for environmental studies, and associated limitations.

3.1. RNA editing

RNA editing involves post-transcriptional modifications that alter RNA sequences, such as adenosine-to-inosine (A-to-I) or cytosine-to-uracil (C-to-U) changes. These modifications enable rapid adaptation to environmental conditions without altering the underlying genome (Krüttner & Caroni, 2019; Nie et al., 2020; Yablonovitch, Deng, Jacobson, & Li, 2017; A. Zhang, Jiang, Zhang, Wang, & Zhang, 2019) and therefore provide insights into adaptive mechanisms and stress responses that are invisible in conventional mRNA abundance studies. For instance, in Arabidopsis, reduced C-to-U RNA editing rates in mitochondrial and chloroplast genes have been observed under heat or cold stress (Chu & Wei, 2020). However, detecting editing events requires high sequencing depth and advanced computational tools. Given the continuously dropping sequencing costs, this still holds a promising avenue for future research and in future eRNA studies, RNA editing could be applied to track real-time adaptation in communities.

3.2. RNA methylation

During RNA methylation, methyl groups are added to certain nucleotides in an RNA molecule. The most common type of methylation, N6-methyladenosine (m6A), regulates RNA stability, translation, and localization (Yue, Liu, & He, 2015). Studying methylation requires advanced techniques like specialized sequencing, e.g., m6A-CLIP, MeRIP-seq and m6A-LAIC-seq , which can be resource-intensive (McIntyre et al., 2020; Owens, Zhang, & Liu, 2021). However, recent advancements in these techniques have improved the detection of m6A modifications in low-abundance RNA samples, making them highly valuable for environmental research (Shabani, Dresselhaus, & Dukowic-Schulze, 2022; Xiao et al., 2023). M6A has recently revealed to be a key molecular response to various environmental stressors such as hypoxia and

changes in temperature or salinity (Ahi & Singh, 2024; Hu et al., 2021; Y. J. Wang et al., 2021). Hence, studying methylation patterns received from environmental samples could reveal either individual species or entire communities reacting to environmental stressors.

3.3. Alternative splicing

Alternative splicing is a post-transcriptional process that enables a single gene to produce multiple mRNA isoforms by selectively including or excluding specific exons during RNA maturation. This mechanism significantly enhances transcriptomic and proteomic diversity, influencing various cellular functions and adaptive responses (Kiran Mandadi et al., 2022; Singh & Ahi, 2022; Verta & Jacobs, 2022). The analysis of alternative splicing provides insights into how organisms and communities modulate gene expression in response to environmental fluctuations, such as temperature changes and pollutants (Bernatchez, Ferchaud, Berger, Venney, & Xuereb, 2023; Liu, Guo, Xu, Liu, & Yan, 2022a; Salisbury, Delgado, & Dalziel, 2021; Steward, de Jong, Oostra, & Wheat, 2022; Verta & Jacobs, 2022; B. Zhao et al., 2024). The detection of diverse spliced isoforms can reveal molecular strategies employed by organisms to rapidly adapt to their habitats (Kiran Mandadi et al., 2022) or interact with other organisms (Betz et al., 2024; Legüe, Aguila, & Calixto, 2021). While conventional methods quantify overall gene expression levels, they may overlook the functional diversity generated by different mRNA isoforms. For instance, specific isoforms can encode proteins with distinct, even opposing, functions, playing critical roles in stress responses and developmental processes (Staiger & Brown, 2013). However, studying alternative splicing in complex environmental samples presents challenges. Accurate identification of isoforms requires high sequencing depth and advanced computational tools, especially when dealing with mixed-species samples where reference genomes may be incomplete or unavailable. Additionally, RNA fragments long enough to identify the exact isoform, are required to be available in the eRNA sample. Also, the functional understanding of novel isoforms is often limited, complicating the interpretation of their ecological significance. Despite these challenges, focusing on alternative splicing in eRNA research holds promise for uncovering how species communities and ecosystems dynamically adjust to environmental pressures. For example, high rates of alternatively spliced genes being expressed under temperature salt stress have been described in plants (Ding et al., 2014; Liu, Guo, Xu, Liu, & Yan, 2022b), therefore the detection of such alternative splicing variants from eRNA samples could reveal plant communities experiencing such environmental stressors.

3.4. RNA pseudouridylation

Pseudouridylation, the conversion of uridine to pseudouridine (Ψ) in RNA, is a prevalent RNA modification that enhances RNA stability, folding, and functionality (B. S. Zhao & He, 2014). Pseudouridylation plays essential roles in RNA processing, translation, and cellular stress responses (L. Sun et al., 2019; Z. Wang et al., 2022). In environmental contexts, it likely enables organisms to stabilize RNA under harsh or fluctuating conditions such as extreme temperatures, high salinity, or oxidative stress, contributing to molecular resilience (Adamiec & Luciński, 2024; Niu & Liu, 2023). Pseudouridylation provides insights into post-transcriptional regulatory mechanisms that stabilize and adapt RNA to environmental challenges (Khan et al., 2023). Its ability to enhance RNA performance under stress conditions highlights its role in organismal survival (Adamiec & Luciński, 2024). However, detecting pseudouridylation in environmental samples remains technically challenging. Methods such as pseudouridine sequencing (Ψ-seq) and mass spectrometry are required to map pseudouridine sites accurately, but these techniques are resource-intensive and not yet widely applied to environmental RNA studies (Begik et al., 2021; Zaringhalam & Papavasiliou, 2016). Detecting pseudouridylation from environmental samples could illuminate whether and how species communities adapt to extreme environments.

3.5. RNA tailing and polyadenylation

RNA tailing is a process in which a string of nucleotides is added to the 3' end of mRNA molecules with polyadenylation being the addition of adenine nucleotides. The addition of those nucleotides influence RNA stability, transport and degradation (Roux et al., 2024; Tudek et al., 2021). Polyadenylation and other variants in RNA tailing, including uridylation, have been observed in diverse organisms, indicating their roles in RNA turnover and stress response (Roux et al., 2024; Wu, Wang, Wu, Hong, & Li, 2020; Zhou & Li, 2023). RNA tailing studies offer insights into RNA stability and degradation, aspects that are overlooked in mRNA abundance analyses (Chou et al., 2015). A good understanding of RNA dynamics such as eRNA production, persistence and degradation is crucial for any inference drawn on eRNA data (Kagzi et al., 2023; Wood et al., 2020) and therefore the influence of RNA tailing on eRNA stability is a key factor to consider. However, the transient nature of RNA tails and their variability across species make them challenging to study and they require tailored sequencing and bioinformatics approaches (M. Chen et al., 2020; Ye, Long, Ji, Li, & Wu, 2018). In future eRNA research, studying the influence of RNA tailing onto eRNA persistence will expand our understanding of eRNA stability in and detection probabilities from environmental samples.

4. Regulatory connections between RNA processes and RNA types

The RNA processes described above can influence the various RNA types discussed earlier. While the full network of interactions between RNA types and RNA modifications within cellular metabolism is beyond the scope of this work, we summarize the key connections between RNA processes and RNA types outlined in this manuscript here and in Table 3.

RNA editing: In mRNAs and lncRNAs, editing can generate alternative isoforms or influence stability. For piRNAs and sncRNAs, editing may fine-tune their target specificity, potentially affecting stress or developmental responses (Picardi, D'Erchia, Gallo, Montalvo, & Pesole, 2014).

RNA methylation: N6-methyladenosine (m6A) affects mRNAs, lncRNAs, sncRNAs, tRNAs, tRFs and circRNAs, marking them for degradation or protection (Motorin & Helm, 2022).

Alternative splicing: While primarily affecting mRNAs and lncRNAs, evidence suggests it also influences circRNA formation (X. O. Zhang et al., 2016). The resulting transcript variants, or isoforms can have differing functions and mediate responses to environmental triggers.

RNA pseudouridylation: This process is particularly relevant for rRNAs, tRNAs, and sncRNAs and affects stability, folding, and functionality of these transcripts (De Zoysa & Yu, 2017).

Polyadenylation and RNA tailing: These processes regulate mRNAs, lncRNAs, and even some vsiRNAs which can either stabilize transcripts or mark them for decay (Yu & Kim, 2020). The poly(A) status of these RNAs influences their persistence outside the cell, as well, thereby affecting their detectability and usefulness in eRNA surveys (Chou et al., 2015; Kagzi et al., 2023; Wood et al., 2020).

5. Overall potential for biodiversity and conservation research

The primary strength of eRNA research lies in its ability to non-invasively infer the physiological state of organisms, going beyond presence–absence data. In addition to mRNA, the RNA types and processes discussed above hold significant potential for monitoring stress responses, developmental stages, pathogen infections and adaptive processes. Most of these have been best studied in the context of stress response, particularly to thermal stress and toxin exposure - conditions that are highly relevant for assessing species and community health in natural environments. These stressors are also among the most pressing conservation concerns, as climate change is projected to alter thermal regimes globally (Burrows et al., 2011; Diffenbaugh & Field, 2013; Maberly et al., 2020), and pollution from domestic and industrial sources is a persisting major threat to biodiversity (Bernhardt, Rosi, & Gessner, 2017; Groh, vom Berg, Schirmer, & Tlili, 2022; Sigmund et al., 2023). Furthermore, the detection of ongoing viral or other pathogen infections, along with corresponding immune responses, represents an additional promising

area that could be addressed through eRNA analysis of these diverse RNA types and processes. This approach would enable non-invasive, large-scale disease monitoring at the ecosystem level and at a rapid pace. Several of these RNA molecules may also enable the non-invasive detection of developmental stages, offering key insights into population viability by assessing reproductive activity and age structure. Finally, the potential of some RNA types to reflect real-time or transgenerational adaptation to environmental stressors could provide critical information on both adaptive mechanisms and the resilience of species and communities under changing environmental conditions. Taken together, among the RNA types and processes discussed, we consider rRNA, microRNAs, and RNA methylation to be particularly promising for future eRNA research and for the research questions discussed. This is due to their relatively well-understood biological functions, the availability of advanced analytical methodologies, and/or their comparatively high stability, which enhances their persistence in environmental samples over time.

6. Current limitations and future directions

Functional understanding of RNA types and RNA processes: While holding great potential, compared to mRNA, our understanding of the functions of the RNA types and processes discussed here remains relatively limited. Much of the current knowledge has been derived from studies in microorganisms, while the roles of these RNAs in macroorganisms, particularly in non-model species, are still poorly understood. Therefore, the integration of these molecules into eRNA research requires additional research of their in vivo functions across a broader range of organisms. Figure 2c indicates our estimation of current applicability of the different RNA types and processes for micro- and macro-organisms.

Varying eRNA persistence in the environment: Aside from the required basic knowledge of RNA function, their persistence in the environment is a crucial factor to consider. Many of the analytical approaches discussed here require reliable quantification of the RNA molecules, at least relative to reference transcripts (e.g., housekeeping genes). When RNA is released into the environment, it is subjected to degradation, a process likely to differ between RNA types. This differential RNA persistence directly affects the abundance of RNA molecules in eRNA samples. As this must be accounted for in data interpretation, a better understanding of persistence times and degradation rates of the different RNA molecules is essential. In Figure 2d we give a speculative overview of eRNA persistence across RNA types and processes, but this requires verification by experimental research.

Distinguishing between organismal and extra-organismal RNA: Also heavily linked to eRNA degradation, a key consideration in eRNA research is whether RNA is located within intact cells (organismal RNA) or

freely present in the environment (extra-organismal RNA) (Figure 2d). Organismal RNA is typically more stable and better reflects of living biodiversity, while extra-organismal RNA is more susceptible to degradation from environmental stressors (Jo, Tsuri, Hirohara, & Yamanaka, 2023) and might indicate species presence or activity of species that are not present in the sampled environment (any more). However, techniques like size-selective filtration or differential centrifugation could help to distinguish these RNA pools in an eRNA sample (Nigro et al., 2021).

Taxonomic annotation of eRNA molecules: One of the major challenges in extended eRNA research is determining the taxonomic origin of RNA molecules from mixed eRNA samples, as sequence conservation and sequence length varies drastically among RNA type. Some of the RNA molecules described exhibit high sequence conservation, resulting in genetic resolutions too low to distinguish closely related species. One way to address this is to shift ecological inference from the species level to a higher taxonomic level, especially when the observed function is known to be conserved across members of that group. This approach could enable a more holistic interpretation of the physiological state of entire species communities. However, it also carries the risk that variation in the physiological states of individual species may be masked by those of other, potentially more abundant, species. The taxonomic annotation of RNA (and DNA) sequences is furthermore highly dependent on comprehensive reference databases. Expanding these databases is an ongoing global effort in eDNA and barcoding efforts (Blackman et al., 2023; Gostel & Kress, 2022; Marques et al., 2021), but most of the current focus lies on individual genetic markers used for barcoding or eDNA metabarcoding. Broadening RNA sequence databases beyond conventional barcode and metabarcoding markers will require extensive transcriptome-level sequencing across a wide range of micro- and macroorganisms. However, given the current pace at which sequence data is being generated, we are optimistic that these resources will continue to expand rapidly.

Need for feasibility assessment of emerging RNA techniques in ecological contexts: Several advanced molecular techniques discussed in this manuscript, such as SHAPE-seq, m6A-CLIP, and Ψ-seq, offer exciting potential to expand the scope of eRNA research by enabling the study of RNA structure and modifications. However, many of these methods remain technically demanding, costly, and currently impractical for routine use in most ecology-focused laboratories. While we attempted to summarize cost and workload under "sequencing feasibility" in Figure 2b), systematic evaluations of these techniques in the context of large-scale environmental studies are still lacking. Such evaluations need to assess key factors including cost-effectiveness, technical complexity, input material requirements, and robustness

when working with degraded or mixed-environmental samples. This is essential for determining their practical value and overall feasibility in advancing eRNA research.

Conclusion

Exploring alternative RNA types and RNA processes in eRNA samples can help answer questions relevant to ecology and conservation that mRNA alone cannot. Most dominantly, non-coding RNAs and RNA modifications can provide additional information on whether species communities currently respond to environmental stressors such as temperature shifts and pollution, detect ongoing pathogenic infections and infer population viability and resilience. However, realizing their full potential in eRNA research is not without challenges. Many alternative RNA types require specialized or costly sequencing techniques, conserved and short RNA sequences hinder taxonomic resolution, and few genetic reference data complicate taxonomic identification. Varying rates of RNA degradation among RNA types can obscure biological signals, and low-abundance RNAs may be hard to detect. Despite these hurdles, progress is accelerating. Sequencing costs are dropping, and new methods are being developed to capture rare or degraded RNAs. For taxonomic inference, higher-level classifications (e.g., class or family) may be sufficient when function is conserved. Alternatively, community-level RNA profiling may offer ecological insights without relying on species-level resolution and reference databases aside from conventional (meta-)barcoding markers continue to expand. Future research needs to focus on a better functional understanding of the RNA function beyond mRNA, developing approaches to distinguish extra- from intra-cellular RNA in eRNA samples, expand the knowledge of RNA persistence across RNA types, and systematically assess feasibility of specialized sequencing techniques.

References:

Abduljalil, J. M. (2018). Bacterial riboswitches and RNA thermometers: Nature and contributions to pathogenesis. *Non-Coding RNA Research*, *3*(2), 54–63. https://doi.org/10.1016/J.NCRNA.2018.04.003

Abe, T., Inokuchi, H., Yamada, Y., Muto, A., Iwasaki, Y., & Ikemura, T. (2014). TRNADB-CE: TRNA gene database well-timed in the era of big sequence data. *Frontiers in Genetics*, *5*(MAY), 83271. https://doi.org/10.3389/FGENE.2014.00114/BIBTEX

Abo-Al-Ela, H. G., & Faggio, C. (2021). MicroRNA-mediated stress response in bivalve species. *Ecotoxicology and Environmental Safety*, *208*, 111442. https://doi.org/10.1016/J.ECOENV.2020.111442

Adamiec, M., & Luciński, R. (2024). The Roles of RNA Modifications in Regulating Chloroplast Performance and Photosynthesis Efficiency. *International Journal of Molecular Sciences 2024, Vol. 25, Page 11912*, *25*(22), 11912. https://doi.org/10.3390/IJMS252211912

Adamo, M., Voyron, S., Chialva, M., Marmeisse, R., & Girlanda, M. (2020). Metabarcoding on both environmental DNA and RNA highlights differences between fungal communities sampled in different habitats. *PLOS ONE*, *15*(12), e0244682. https://doi.org/10.1371/JOURNAL.PONE.0244682

Ahi, E. P., & Singh, P. (2024). An emerging orchestrator of ecological adaptation: m6A regulation of post-transcriptional mechanisms. *Molecular Ecology*, 17545. https://doi.org/10.1111/MEC.17545

An, H. E., Mun, M. H., & Kim, C. B. (2023). Metabarcoding by Combining Environmental DNA with Environmental RNA to Monitor Fish Species in the Han River, Korea. *Fishes*, *8*(11), 550. https://doi.org/10.3390/FISHES8110550/S1

Asgari, S. (2017). RNA as a means of inter-species communication and manipulation: Progresses and shortfalls. *RNA Biology*, *14*(4), 389–390. https://doi.org/10.1080/15476286.2017.1306172

Baldridge, K. C., & Contreras, L. M. (2014). Functional implications of ribosomal RNA methylation in response to environmental stress. *Critical Reviews in Biochemistry and Molecular Biology*, *49*(1), 69–89. https://doi.org/10.3109/10409238.2013.859229

Begik, O., Lucas, M. C., Pryszcz, L. P., Ramirez, J. M., Medina, R., Milenkovic, I., … Novoa, E. M. (2021). Quantitative profiling of pseudouridylation dynamics in native RNAs with nanopore sequencing. *Nature Biotechnology 2021 39:10*, *39*(10), 1278–1291. https://doi.org/10.1038/s41587-021-00915-6

Belfort, M. (2017). Mobile self-splicing introns and inteins as environmental sensors. *Current Opinion in Microbiology*, *38*, 51. https://doi.org/10.1016/J.MIB.2017.04.003

Belicard, T., Jareosettasin, P., & Sarkies, P. (2018). The piRNA pathway responds to environmental signals to establish intergenerational adaptation to stress. *BMC Biology*, *16*(1), 1–14. https://doi.org/10.1186/S12915-018-0571-Y/FIGURES/6

Bernatchez, L., Ferchaud, A. L., Berger, C. S., Venney, C. J., & Xuereb, A. (2023). Genomics for monitoring and understanding species responses to global climate change. *Nature Reviews Genetics 2023 25:3*, *25*(3), 165–183. https://doi.org/10.1038/s41576-023-00657-y

Bernhardt, E. S., Rosi, E. J., & Gessner, M. O. (2017). Synthetic chemicals as agents of global change. *Frontiers in Ecology and the Environment*, *15*(2), 84–90. https://doi.org/10.1002/FEE.1450

Betz, R., Heidt, S., Figueira-Galán, D., Hartmann, M., Langner, T., & Requena, N. (2024). Alternative splicing regulation in plants by SP7-like effectors from symbiotic arbuscular mycorrhizal fungi. *Nature Communications 2024 15:1*, *15*(1), 1–21. https://doi.org/10.1038/s41467-024-51512-5

Biggar, K. K., & Storey, K. B. (2018). Functional impact of microRNA regulation in models of extreme stress adaptation. *Journal of Molecular Cell Biology*, *10*(2), 93–101. https://doi.org/10.1093/JMCB/MJX053

Björk, G. R., & Hagervall, T. G. (2014). Transfer RNA Modification: Presence, Synthesis, and Function. *EcoSal Plus*, *6*(1). https://doi.org/10.1128/ECOSALPLUS.ESP-0007-2013

Blackman, R. C., Walser, J. C., Rüber, L., Brantschen, J., Villalba, S., Brodersen, J., … Altermatt, F. (2023). General principles for assignments of communities from eDNA: Open versus closed taxonomic databases. *Environmental DNA*, *5*(2), 326–342. https://doi.org/10.1002/EDN3.382

Blazewicz, S. J., Barnard, R. L., Daly, R. A., & Firestone, M. K. (2013). Evaluating rRNA as an indicator of microbial activity in environmental communities: limitations and uses. *The ISME Journal*, *7*(11), 2061–2068. https://doi.org/10.1038/ISMEJ.2013.102

Burrows, M. T., Schoeman, D. S., Buckley, L. B., Moore, P., Poloczanska, E. S., Brander, K. M., … Richardson, A. J. (2011). The pace of shifting climate in marine and terrestrial ecosystems. *Science*, *334*(6056), 652–655. https://doi.org/10.1126/SCIENCE.1210288/SUPPL_FILE/BURROWS.SOM.PDF

Carrington, J. C., & Ambros, V. (2003). Role of MicroRNAs in Plant and Animal Development. *Science*, *301*(5631), 336–338. https://doi.org/10.1126/SCIENCE.1085242

Casier, K., Boivin, A., Carré, C., & Teysset, L. (2019). Environmentally-Induced Transgenerational Epigenetic Inheritance: Implication of PIWI Interacting RNAs. *Cells 2019, Vol. 8, Page 1108*, *8*(9), 1108. https://doi.org/10.3390/CELLS8091108

Casier, K., Delmarre, V., Gueguen, N., Hermant, C., Viodé, E., Vaury, C., … Boivin, A. (2019). Environmentally-induced epigenetic conversion of a piRNA cluster. *ELife*, *8*. https://doi.org/10.7554/ELIFE.39842

Catalan-Moreno, A., Cela, M., Menendez-Gil, P., Irurzun, N., Caballero, C. J., Caldelari, I., & Toledo-Arana, A. (2021). RNA thermoswitches modulate Staphylococcus aureus adaptation to ambient temperatures. *Nucleic Acids Research*, *49*(6), 3409–3426. https://doi.org/10.1093/NAR/GKAB117

Chan, P. P., Lin, B. Y., Mak, A. J., & Lowe, T. M. (2021). tRNAscan-SE 2.0: improved detection and functional classification of transfer RNA genes. *Nucleic Acids Research*, *49*(16), 9077–9096. https://doi.org/10.1093/NAR/GKAB688

Chen, M., Ji, G., Fu, H., Lin, Q., Ye, C., Ye, W., … Wu, X. (2020). A survey on identification and quantification of alternative polyadenylation sites from RNA-seq data. *Briefings in Bioinformatics*, *21*(4), 1261–1276. https://doi.org/10.1093/BIB/BBZ068

Chen, Xuebing, Xu, H., Lin, Y., & Zhu, B. (2024). Forensic stability evaluation of selected miRNA and circRNA markers in human bloodstained samples exposed to different environmental conditions. *Forensic Science International*, *362*, 112148. https://doi.org/10.1016/J.FORSCIINT.2024.112148

Chen, Xuemei, & Rechavi, O. (2021). Plant and animal small RNA communications between cells and organisms. *Nature Reviews Molecular Cell Biology 2021 23:3*, *23*(3), 185–203. https://doi.org/10.1038/s41580-021-00425-y

Chi, X., Wang, Z., Wang, Y., Liu, Z., Wang, H., & Xu, B. (2023). Cross-Kingdom Regulation of Plant-Derived miRNAs in Modulating Insect Development. *International Journal of Molecular Sciences 2023, Vol. 24, Page 7978*, *24*(9), 7978. https://doi.org/10.3390/IJMS24097978

Cholet, F., Ijaz, U. Z., & Smith, C. J. (2019). Differential ratio amplicons (Ramp) for the evaluation of RNA integrity extracted from complex environmental samples. *Environmental Microbiology*, *21*(2), 827–844. https://doi.org/10.1111/1462-2920.14516

607 Chou, M. Te, Han, B. W., Hsiao, C. P., Zamore, P. D., Weng, Z., & Hung, J. H. (2015). Tailor: a
608 computational framework for detecting non-templated tailing of small silencing RNAs. *Nucleic*
609 *Acids Research*, *43*(17), e109–e109. https://doi.org/10.1093/NAR/GKV537
610 Chu, D., & Wei, L. (2020). Reduced C-to-U RNA editing rates might play a regulatory role in stress
611 response of Arabidopsis. *Journal of Plant Physiology*, *244*, 153081.
612 https://doi.org/10.1016/J.JPLPH.2019.153081
613 Cristescu, M. E. (2019). Can Environmental RNA Revolutionize Biodiversity Science? *Trends in Ecology*
614 *and Evolution*, *34*(8), 694–697. https://doi.org/10.1016/j.tree.2019.05.003
615 Dang, Y., Yan, L., Hu, B., Fan, X., Ren, Y., Li, R., … Qiao, J. (2016). Tracing the expression of circular RNAs
616 in human pre-implantation embryos. *Genome Biology*, *17*(1), 1–15.
617 https://doi.org/10.1186/S13059-016-0991-3/FIGURES/6
618 De Zoysa, M. D., & Yu, Y. T. (2017). Posttranscriptional RNA Pseudouridylation. *Enzymes*, *41*, 151–167.
619 https://doi.org/10.1016/BS.ENZ.2017.02.001
620 Diffenbaugh, N. S., & Field, C. B. (2013). Changes in ecologically critical terrestrial climate conditions.
621 *Science*, *341*(6145), 486–492.
622 https://doi.org/10.1126/SCIENCE.1237123/SUPPL_FILE/DIFFENBAUGH-SM.PDF
623 Ding, F., Cui, P., Wang, Z., Zhang, S., Ali, S., & Xiong, L. (2014). Genome-wide analysis of alternative
624 splicing of pre-mRNA under salt stress in Arabidopsis. *BMC Genomics*, *15*(1), 1–14.
625 https://doi.org/10.1186/1471-2164-15-431/FIGURES/6
626 Drula, R., Braicu, C., & Neagoe, I. B. (2024). Current advances in circular RNA detection and investigation
627 methods: Are we running in circles? *Wiley Interdisciplinary Reviews: RNA*, *15*(3), e1850.
628 https://doi.org/10.1002/WRNA.1850
629 Ebbesen, K. K., Hansen, T. B., & Kjems, J. (2017). Insights into circular RNA biology. *RNA Biology*, *14*(8),
630 1035–1045. https://doi.org/10.1080/15476286.2016.1271524
631 Endres, L., Dedon, P. C., & Begley, T. J. (2015). Codon-biased translation can be regulated by wobble-
632 base tRNA modification systems during cellular stress responses. *RNA Biology*, *12*(6), 603–614.
633 https://doi.org/10.1080/15476286.2015.1031947
634 Fahad, M., Tariq, L., Muhammad, S., & Wu, L. (2024). Underground communication: Long non-coding
635 RNA signaling in the plant rhizosphere. *Plant Communications*, *5*(7).
636 https://doi.org/10.1016/J.XPLC.2024.100927/ASSET/7C14E99E-4119-4C4C-A1E1-
637 10E89CCA3A52/MAIN.ASSETS/GR4.JPG
638 Fischer, J. W., & Leung, A. K. L. (2017). CircRNAs: a regulator of cellular stress. *Critical Reviews in*
639 *Biochemistry and Molecular Biology*, *52*(2), 220–233.
640 https://doi.org/10.1080/10409238.2016.1276882
641 Frommer, J., Appel, B., & Müller, S. (2015). Ribozymes that can be regulated by external stimuli. *Current*
642 *Opinion in Biotechnology*, *31*, 35–41. https://doi.org/10.1016/J.COPBIO.2014.07.009
643 Gao, P., Fan, K., Zhang, G., Yin, X., Jia, C., & Tian, H. (2023). Coal-mining subsidence changed distribution
644 of the microbiomes and their functional genes in a farmland. *Journal of Basic Microbiology*, *63*(5),
645 542–557. https://doi.org/10.1002/JOBM.202200582
646 Gelsinger, D. R., Uritskiy, G., Reddy, R., Munn, A., Farney, K., & DiRuggiero, J. (2020). Regulatory
647 Noncoding Small RNAs Are Diverse and Abundant in an Extremophilic Microbial Community.
648 *MSystems*, *5*(1). https://doi.org/10.1128/MSYSTEMS.00584-19/SUPPL_FILE/MSYSTEMS.00584-19-
649 SF007.TIF

650 Giroux, M. S., Reichman, J. R., Langknecht, T., Burgess, R. M., & Ho, K. T. (2022). Environmental RNA as a
651 Tool for Marine Community Biodiversity Assessments. *Scientific Reports 2022 12:1*, *12*(1), 1–13.
652 https://doi.org/10.1038/s41598-022-22198-w

653 Giroux, M. S., Reichman, J. R., Langknecht, T., Burgess, R. M., & Ho, K. T. (2023). Using eRNA/eDNA
654 metabarcoding to detect community-level impacts of nanoplastic exposure to benthic estuarine
655 ecosystems. *Environmental Pollution (Barking, Essex : 1987)*, *338*.
656 https://doi.org/10.1016/J.ENVPOL.2023.122650

657 Gostel, M. R., & Kress, W. J. (2022). The Expanding Role of DNA Barcodes: Indispensable Tools for
658 Ecology, Evolution, and Conservation. *Diversity 2022, Vol. 14, Page 213*, *14*(3), 213.
659 https://doi.org/10.3390/D14030213

660 Greco, M., Lejzerowicz, F., Reo, E., Caruso, A., Maccotta, A., Coccioni, R., … Frontalini, F. (2022).
661 Environmental RNA outperforms eDNA metabarcoding in assessing impact of marine pollution: A
662 chromium-spiked mesocosm test. *Chemosphere*, *298*, 134239.
663 https://doi.org/10.1016/J.CHEMOSPHERE.2022.134239

664 Groh, K., vom Berg, C., Schirmer, K., & Tlili, A. (2022). Anthropogenic Chemicals As Underestimated
665 Drivers of Biodiversity Loss: Scientific and Societal Implications. *Environmental Science and*
666 *Technology*, *56*(2), 707–710.
667 https://doi.org/10.1021/ACS.EST.1C08399/ASSET/IMAGES/LARGE/ES1C08399_0003.JPEG

668 Gualtieri, C., Leonetti, P., & Macovei, A. (2020). Plant miRNA Cross-Kingdom Transfer Targeting Parasitic
669 and Mutualistic Organisms as a Tool to Advance Modern Agriculture. *Frontiers in Plant Science*, *11*,
670 531283. https://doi.org/10.3389/FPLS.2020.00930/BIBTEX

671 Hayden, E. J., & Wagner, A. (2012). Environmental change exposes beneficial epistatic interactions in a
672 catalytic RNA. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1742), 3418–3425.
673 https://doi.org/10.1098/RSPB.2012.0956

674 Heintz-Buschart, A., Yusuf, D., Kaysen, A., Etheridge, A., Fritz, J. V., May, P., … Wilmes, P. (2018). Small
675 RNA profiling of low biomass samples: Identification and removal of contaminants. *BMC Biology*,
676 *16*(1), 1–11. https://doi.org/10.1186/S12915-018-0522-7/FIGURES/6

677 Hoffmann, A., Lorenz, C., Fallmann, J., Wolff, P., Lechner, A., Betat, H., … Stadler, P. F. (2024).
678 Temperature-Dependent tRNA Modifications in Bacillales. *International Journal of Molecular*
679 *Sciences*, *25*(16). https://doi.org/10.3390/IJMS25168823

680 Hu, J., Cai, J., Park, S. J., Lee, K., Li, Y., Chen, Y., … Kang, H. (2021). N6-Methyladenosine mRNA
681 methylation is important for salt stress tolerance in Arabidopsis. *The Plant Journal*, *106*(6), 1759–
682 1775. https://doi.org/10.1111/TPJ.15270

683 Huber, S. M., Leonardi, A., Dedon, P. C., & Begley, T. J. (2019). The Versatile Roles of the tRNA
684 Epitranscriptome during Cellular Responses to Toxic Exposures and Environmental Stress. *Toxics*,
685 *7*(1), 17. https://doi.org/10.3390/TOXICS7010017

686 Huo, D., Sun, L., Sun, J., Lin, C., Liu, S., Zhang, L., & Yang, H. (2021). Emerging roles of circRNAs in
687 regulating thermal and hypoxic stresses in Apostichopus japonicus (Echinodermata:
688 Holothuroidea). *Ecotoxicology and Environmental Safety*, *228*, 112994.
689 https://doi.org/10.1016/J.ECOENV.2021.112994

690 Jo, T., Tsuri, K., Hirohara, T., & Yamanaka, H. (2023). Warm temperature and alkaline conditions
691 accelerate environmental RNA degradation. *Environmental DNA*, *5*(5), 836–848.
692 https://doi.org/10.1002/EDN3.334

693 Kagzi, K., Millette, K. L., Littlefair, J. E., Pochon, X., Wood, S. A., Fussmann, G. F., & Cristescu, M. E.

(2023). Assessing the degradation of environmental DNA and RNA based on genomic origin in a metabarcoding context. *Environmental DNA*, *5*(5), 1016–1031. https://doi.org/10.1002/EDN3.437

Kalwan, G., Gill, S. S., Priyadarshini, P., Gill, R., Yadava, Y. K., Yadav, S., … Jain, P. K. (2023). Approaches for identification and analysis of plant circular RNAs and their role in stress responses. *Environmental and Experimental Botany*, *205*, 105099. https://doi.org/10.1016/J.ENVEXPBOT.2022.105099

Khan, A., Hu, Y. D., Khan, S., Aqeel, S. M., Khan, I., Hussain, M., … Xu, G. (2023). Pseudouridine in RNA: Enzymatic Synthesis Mechanisms and Functional Roles in Molecular Biology. *International Journal of Environment, Agriculture and Biotechnology*, *8*(6). https://doi.org/10.22161/ijeab

Kimura, S., Srisuknimit, V., & Waldor, M. K. (2020). Probing the diversity and regulation of tRNA modifications. *Current Opinion in Microbiology*, *57*, 41–48. https://doi.org/10.1016/J.MIB.2020.06.005

Kiran Mandadi, K., Balasubramanian, S., Min, X., Liu pliu, P., Kang Yan, danforthcenterorg, Liu, X.-X., … Yan, K. (2022). Rapid Regulation of Alternative Splicing in Response to Environmental Stresses. *Frontiers in Plant Science*, *13*, 832177. https://doi.org/10.3389/FPLS.2022.832177

Klappenbach, J. A., Dunbar, J. M., & Schmidt, T. M. (2000). rRNA operon copy number reflects ecological strategies of bacteria. *Applied and Environmental Microbiology*, *66*(4), 1328–1333. https://doi.org/10.1128/AEM.66.4.1328-1333.2000/ASSET/4C1C8576-7607-4845-BE02-A577DD20957E/ASSETS/GRAPHIC/AM0401728004.JPEG

Krüttner, S., & Caroni, P. (2019, January 1). m6A-epitranscriptome modulates memory strength. *Cell Research*. Springer Nature. https://doi.org/10.1038/s41422-018-0121-8

Lakshmanan, V., Sujith, T. N., Bansal, D., Shivaprasad, P. V., Palakodeti, D., & Krishna, S. (2021). Comprehensive annotation and characterization of planarian tRNA and tRNA-derived fragments (tRFs). *RNA*, *27*(4), 477–495. https://doi.org/10.1261/RNA.077701.120

Lalande, S., Merret, R., Salinas-Giegé, T., & Drouard, L. (2020). Arabidopsis tRNA-derived fragments as potential modulators of translation. *RNA Biology*, *17*(8), 1137–1148. https://doi.org/10.1080/15476286.2020.1722514

Lasda, E., & Parker, R. (2016). Circular RNAs Co-Precipitate with Extracellular Vesicles: A Possible Mechanism for circRNA Clearance. *PLOS ONE*, *11*(2), e0148407. https://doi.org/10.1371/JOURNAL.PONE.0148407

Legüe, M., Aguila, B., & Calixto, A. (2021). Interspecies RNA Interactome of Pathogen and Host in a Heritable Defensive Strategy. *Frontiers in Microbiology*, *12*, 649858. https://doi.org/10.3389/FMICB.2021.649858/BIBTEX

Leppek, K., & Barna, M. (2019). An rRNA variant to deal with stress. *Nature Microbiology*, *4*(3), 382–383. https://doi.org/10.1038/S41564-019-0396-7

Leung, A. K. L., & Sharp, P. A. (2010). MicroRNA Functions in Stress Responses. *Molecular Cell*, *40*(2), 205–215. https://doi.org/10.1016/J.MOLCEL.2010.09.027/ASSET/B35B01FA-9ED2-4A6E-BE5B-4E1D145A66AF/MAIN.ASSETS/GR5.JPG

Li, M. L., Weng, K. F., Shih, S. R., & Brewer, G. (2016). The evolving world of small RNAs from RNA viruses. *Wiley Interdisciplinary Reviews: RNA*, *7*(5), 575–588. https://doi.org/10.1002/WRNA.1351

Liu, X. X., Guo, Q. H., Xu, W. B., Liu, P., & Yan, K. (2022a). Rapid Regulation of Alternative Splicing in Response to Environmental Stresses. *Frontiers in Plant Science*, *13*, 832177. https://doi.org/10.3389/FPLS.2022.832177/BIBTEX

737 Liu, X. X., Guo, Q. H., Xu, W. B., Liu, P., & Yan, K. (2022b). Rapid Regulation of Alternative Splicing in
738 Response to Environmental Stresses. *Frontiers in Plant Science*, *13*, 832177.
739 https://doi.org/10.3389/FPLS.2022.832177/BIBTEX

740 Llave, C. (2010). Virus-derived small interfering RNAs at the core of plant-virus interactions. *Trends in*
741 *Plant Science*, *15*(12), 701–707. https://doi.org/10.1016/J.TPLANTS.2010.09.001/ASSET/638434B6-
742 CD9A-4C65-A125-25A6E6F44769/MAIN.ASSETS/GR1.SML

743 Loh, E., Righetti, F., Eichner, H., Twittenhoff, C., & Narberhaus, F. (2018). RNA Thermometers in Bacterial
744 Pathogens. *Regulating with RNA in Bacteria and Archaea*, 55–73.
745 https://doi.org/10.1128/9781683670247.CH4

746 Lorenz, C., Lünse, C. E., & Mörl, M. (2017). tRNA Modifications: Impact on Structure and Thermal
747 Adaptation. *Biomolecules 2017, Vol. 7, Page 35*, *7*(2), 35. https://doi.org/10.3390/BIOM7020035

748 Lott, S. C., Voigt, K., Lambrecht, S. J., Hess, W. R., & Steglich, C. (2020). A framework for the
749 computational prediction and analysis of non-coding RNAs in microbial environmental populations
750 and their experimental validation. *The ISME Journal*, *14*(8), 1955–1965.
751 https://doi.org/10.1038/S41396-020-0658-7

752 Lu, C., Tej, S. S., Luo, S., Haudenschild, C. D., Meyers, B. C., & Green, P. J. (2005). Genetics: Elucidation of
753 the small RNA component of the transcriptome. *Science*, *309*(5740), 1567–1569.
754 https://doi.org/10.1126/SCIENCE.1114112/SUPPL_FILE/LU.SOM.PDF

755 Maberly, S. C., O'Donnell, R. A., Woolway, R. I., Cutler, M. E. J., Gong, M., Jones, I. D., … Tyler, A. N.
756 (2020). Global lake thermal regions shift under climate change. *Nature Communications 2020 11:1*,
757 *11*(1), 1–9. https://doi.org/10.1038/s41467-020-15108-z

758 Macher, T. H., Arle, J., Beermann, A. J., Frank, L., Hupało, K., Koschorreck, J., … Leese, F. (2024). Is it
759 worth the extra mile? Comparing environmental DNA and RNA metabarcoding for vertebrate and
760 invertebrate biodiversity surveys in a lowland stream. *PeerJ*, *12*(10), e18016.
761 https://doi.org/10.7717/PEERJ.18016/SUPP-15

762 Mandin, P., & Johansson, J. (2020). Feeling the heat at the millennium: Thermosensors playing with fire.
763 *Molecular Microbiology*, *113*(3), 588–592. https://doi.org/10.1111/MMI.14468

764 Margis, R., Eguiluz, M., Guzman, F., Rodrigues, N. F., & Dias-Oliveira, M. (2023). Abiotic Stress-
765 Responsive TRNA-Derived Fragments in *Eugenia Uniflora*: Insights into Regulatory Mechanisms and
766 Adaptation to Drought and Salinity. https://doi.org/10.2139/SSRN.4630771

767 Marques, V., Milhau, T., Albouy, C., Dejean, T., Manel, S., Mouillot, D., & Juhel, J. B. (2021). GAPeDNA:
768 Assessing and mapping global species gaps in genetic databases for eDNA metabarcoding. *Diversity*
769 *and Distributions*, *27*(10), 1880–1892. https://doi.org/10.1111/DDI.13142

770 McIntyre, A. B. R., Gokhale, N. S., Cerchietti, L., Jaffrey, S. R., Horner, S. M., & Mason, C. E. (2020). Limits
771 in the detection of m6A changes using MeRIP/m6A-seq. *Scientific Reports 2020 10:1*, *10*(1), 1–15.
772 https://doi.org/10.1038/s41598-020-63355-3

773 Meng, L., Teerds, K., Tao, J., Wei, H., Jaklofsky, M., Zhao, Z., … Zhang, S. (2020). Characteristics of Circular
774 RNA Expression Profiles of Porcine Granulosa Cells in Healthy and Atretic Antral Follicles.
775 *International Journal of Molecular Sciences 2020, Vol. 21, Page 5217*, *21*(15), 5217.
776 https://doi.org/10.3390/IJMS21155217

777 Meyer, S., Carlson, P. D., & Lucks, J. B. (2017). Characterizing the Structure-Function Relationship of a
778 Naturally Occurring RNA Thermometer. *Biochemistry*, *56*(51), 6629–6638.
779 https://doi.org/10.1021/ACS.BIOCHEM.7B01170/SUPPL_FILE/BI7B01170_SI_001.PDF

780 Miyata, K., Inoue, Y., Amano, Y., Nishioka, T., Yamane, M., Kawaguchi, T., … Honda, H. (2021). Fish

environmental RNA enables precise ecological surveys with high positive predictivity. *Ecological Indicators*, *128*, 107796. https://doi.org/10.1016/J.ECOLIND.2021.107796

Molla-Herman, A., Angelova, M. T., Ginestet, M., Carré, C., Antoniewski, C., & Huynh, J. R. (2020). tRNA Fragments Populations Analysis in Mutants Affecting tRNAs Processing and tRNA Methylation. *Frontiers in Genetics*, *11*, 518949. https://doi.org/10.3389/FGENE.2020.518949/BIBTEX

Motorin, Y., & Helm, M. (2022). RNA nucleotide methylation: 2021 update. *Wiley Interdisciplinary Reviews: RNA*, *13*(1), e1691. https://doi.org/10.1002/WRNA.1691

Murakami, S., Fujishima, K., Tomita, M., & Kanai, A. (2012). Metatranscriptomic analysis of microbes in an oceanfront deep-subsurface hot spring reveals novel small RNAs and type-specific tRNA degradation. *Applied and Environmental Microbiology*, *78*(4), 1015–1022. https://doi.org/10.1128/AEM.06811-11/SUPPL_FILE/AEM6811-11_SUPPLEMENTAL_MATERIAL.PDF

Nawaz, M. Z., & Wang, F. (2022). Meta-omics approaches reveal unique small RNAs exhibited by the uncultured microorganisms dwelling deep-sea hydrothermal sediment in Guaymas Basin. *Archives of Microbiology*, *204*(8), 1–12. https://doi.org/10.1007/S00203-022-03085-4/TABLES/2

Nejat, N., & Mantri, N. (2018). Emerging roles of long non-coding RNAs in plant response to biotic and abiotic stresses. *Critical Reviews in Biotechnology*, *38*(1), 93–105. https://doi.org/10.1080/07388551.2017.1312270

Nie, W., Wang, S., He, R., Xu, Q., Wang, P., Wu, Y., … Chen, G. (2020). A-to-I RNA editing in bacteria increases pathogenicity and tolerance to oxidative stress. *PLOS Pathogens*, *16*(8), e1008740. https://doi.org/10.1371/JOURNAL.PPAT.1008740

Nigro, A., Finardi, A., Ferraro, M. M., Manno, D. E., Quattrini, A., Furlan, R., & Romano, A. (2021). Selective loss of microvesicles is a major issue of the differential centrifugation isolation protocols. *Scientific Reports 2021 11:1*, *11*(1), 1–10. https://doi.org/10.1038/s41598-021-83241-w

Niu, Y., & Liu, L. (2023). RNA pseudouridine modification in plants. *Journal of Experimental Botany*, *74*(21), 6431–6447. https://doi.org/10.1093/JXB/ERAD323

Noll, P., Treinen, C., Müller, S., Senkalla, S., Lilge, L., Hausmann, R., & Henkel, M. (2019). Evaluating temperature-induced regulation of a ROSE-like RNA-thermometer for heterologous rhamnolipid production in Pseudomonas putida KT2440. *AMB Express*, *9*(1), 1–10. https://doi.org/10.1186/S13568-019-0883-5/FIGURES/5

Ou, J., Chen, H., Luan, X., Ju, R., Sun, Y., Zhang, B., … Zhao, W. (2022). Leveraging lncRNA-miRNA-mRNA network to reveal anti-Spiroplasma eriocheiris infection mechanisms in Macrobrachium nipponense. *Aquaculture*, *557*, 738286. https://doi.org/10.1016/J.AQUACULTURE.2022.738286

Owens, M. C., Zhang, C., & Liu, K. F. (2021). Recent technical advances in the study of nucleic acid modifications. *Molecular Cell*, *81*(20), 4116–4136. https://doi.org/10.1016/J.MOLCEL.2021.07.036

Ozata, D. M., Gainetdinov, I., Zoch, A., O'Carroll, D., & Zamore, P. D. (2018). PIWI-interacting RNAs: small RNAs with big functions. *Nature Reviews Genetics 2018 20:2*, *20*(2), 89–108. https://doi.org/10.1038/s41576-018-0073-3

Papastavrou, N., Horning, D. P., & Joyce, G. F. (2024). RNA-catalyzed evolution of catalytic RNA. *Proceedings of the National Academy of Sciences of the United States of America*, *121*(11), e2321592121. https://doi.org/10.1073/PNAS.2321592121/SUPPL_FILE/PNAS.2321592121.SM01.M4V

Peri, G., Gibard, C., Shults, N. H., Crossin, K., & Hayden, E. J. (2022). Dynamic RNA Fitness Landscapes of a Group I Ribozyme during Changes to the Experimental Environment. *Molecular Biology and*

Evolution, *39*(3). https://doi.org/10.1093/MOLBEV/MSAB373

Picardi, E., D'Erchia, A. M., Gallo, A., Montalvo, A., & Pesole, G. (2014). Uncovering RNA editing sites in long non-coding RNAs. *Frontiers in Bioengineering and Biotechnology*, *2*(DEC), 122133. https://doi.org/10.3389/FBIOE.2014.00064/BIBTEX

Popović, M., Fliss, P. S., & Ditzler, M. A. (2015). In vitro evolution of distinct self-cleaving ribozymes in diverse environments. *Nucleic Acids Research*, *43*(14), 7070–7082. https://doi.org/10.1093/NAR/GKV648

Prossliner, T., Agrawal, S., Heidemann, D. F., Sørensen, M. A., & Svenningsen, S. L. (2023). tRNAs Are Stable After All: Pitfalls in Quantification of tRNA from Starved Escherichia coli Cultures Exposed by Validation of RNA Purification Methods. *MBio*, *14*(1). https://doi.org/10.1128/MBIO.02805-22/ASSET/48343D29-4363-4999-8030-69D28B3A5339/ASSETS/IMAGES/MEDIUM/MBIO.02805-22-F008.GIF

Qu, M., Zhao, Y., Zhao, Y., Rui, Q., Kong, Y., & Wang, D. (2019). Identification of long non-coding RNAs in response to nanopolystyrene in Caenorhabditis elegans after long-term and low-dose exposure. *Environmental Pollution*, *255*, 113137. https://doi.org/10.1016/J.ENVPOL.2019.113137

Rashad, S., Al-Mesitef, S., Mousa, A., Zhou, Y., Ando, D., Sun, G., … Niizuma, K. (2024). Translational response to mitochondrial stresses is orchestrated by tRNA modifications. *BioRxiv*, 2024.02.14.580389. https://doi.org/10.1101/2024.02.14.580389

Raza, A., Siddique, K. H. M., & Hu, Z. (2024). Chloroplast gene control: unlocking RNA thermometer mechanisms in photosynthetic systems. *Trends in Plant Science*, *29*(6), 623–625. https://doi.org/10.1016/j.tplants.2024.01.005

Raza, S. H. A., Wijayanti, D., Pant, S. D., Abdelnour, S. A., Hashem, N. M., Amin, A., … Zan, L. (2022). Exploring the physiological roles of circular RNAs in livestock animals. *Research in Veterinary Science*, *152*, 726–735. https://doi.org/10.1016/J.RVSC.2022.09.036

Regmi, R., Penton, C. R., Anderson, J., & Gupta, V. V. S. R. (2022). Do small RNAs unlock the below ground microbiome-plant interaction mystery? *Frontiers in Molecular Biosciences*, *9*, 1017392. https://doi.org/10.3389/FMOLB.2022.1017392/XML/NLM

Roncarati, D., Vannini, A., & Scarlato, V. (2024). Temperature sensing and virulence regulation in pathogenic bacteria. *Trends in Microbiology*, *0*(0). https://doi.org/10.1016/J.TIM.2024.07.009/ASSET/6CF0F4B0-8684-4D74-B1B9-938151969393/MAIN.ASSETS/GR3.JPG

Roux, C., Ramos-Hue, M., Audonnet, M., Duviau, M.-P., Nouaille, S., Carpousis, A. J., … Paul Babitzke, E. (2024). RNA stability is regulated by both RNA polyadenylation and ATP levels, linking RNA and energy metabolisms in Escherichia coli. *MBio*. https://doi.org/10.1128/MBIO.02680-24

Saikia, M., Krokowski, D., Guan, B. J., Ivanov, P., Parisien, M., Hu, G. F., … Hatzoglou, M. (2012). Genome-wide identification and quantitative analysis of cleaved tRNA fragments induced by cellular stress. *Journal of Biological Chemistry*, *287*(51), 42708–42725. https://doi.org/10.1074/JBC.M112.371799/ASSET/56A1DC4C-5D22-423C-B2C9-66E2FF192782/MAIN.ASSETS/FX1.JPG

Salisbury, S. J., Delgado, M. L., & Dalziel, A. C. (2021). Alternative splicing: An overlooked mechanism contributing to local adaptation? *Molecular Ecology*, *30*(20), 4951–4954. https://doi.org/10.1111/MEC.16177

Sarshar, M., Scribano, D., Palamara, A. T., Ambrosi, C., & Masotti, A. (2022). The Acinetobacter baumannii model can explain the role of small non-coding RNAs as potential mediators of host-

pathogen interactions. *Frontiers in Molecular Biosciences*, *9*, 1088783. https://doi.org/10.3389/FMOLB.2022.1088783/BIBTEX

Schauss, J., Kundu, A., Fingerhut, B. P., & Elsaesser, T. (2021). Magnesium Contact Ions Stabilize the Tertiary Structure of Transfer RNA: Electrostatics Mapped by Two-Dimensional Infrared Spectra and Theoretical Simulations. *Journal of Physical Chemistry B*, *125*(3), 740–747. https://doi.org/10.1021/ACS.JPCB.0C08966/ASSET/IMAGES/LARGE/JP0C08966_0005.JPEG

Schwartz, M. H., Wang, H., Pan, J. N., Clark, W. C., Cui, S., Eckwahl, M. J., … Eren, A. M. (2018). Microbiome characterization by high-throughput transfer RNA sequencing and modification analysis. *Nature Communications 2018 9:1*, *9*(1), 1–13. https://doi.org/10.1038/s41467-018-07675-z

Shabani, D., Dresselhaus, T., & Dukowic-Schulze, S. (2022). Profiling m6A RNA Modifications in Low Amounts of Plant Cells Using Maize Meiocytes. *Methods in Molecular Biology*, *2484*, 313–331. https://doi.org/10.1007/978-1-0716-2253-7_21

Sharma, M., Zhang, H., Ehrenkaufer, G., & Singh, U. (2023). Stress Response in Entamoeba histolytica Is Associated with Robust Processing of tRNA to tRNA Halves. *MBio*, *14*(2). https://doi.org/10.1128/MBIO.03450-22/ASSET/83CDDEE9-4F14-4537-89ED-30240B9E0040/ASSETS/IMAGES/MEDIUM/MBIO.03450-22-F007.GIF

Sigmund, G., Ågerstrand, M., Antonelli, A., Backhaus, T., Brodin, T., Diamond, M. L., … Groh, K. J. (2023). Addressing chemical pollution in biodiversity research. *Global Change Biology*, *29*(12), 3240–3255. https://doi.org/10.1111/GCB.16689

Singh, P., & Ahi, E. P. (2022). The importance of alternative splicing in adaptive evolution. *Molecular Ecology*, *31*(7), 1928–1938. https://doi.org/10.1111/mec.16377

Somero, G. N. (2018). RNA thermosensors: How might animals exploit their regulatory potential? *Journal of Experimental Biology*, *221*(4). https://doi.org/10.1242/JEB.162842/33922

Song, Y., & Zhang, D. (2017). The Role of Long Noncoding RNAs in Plant Stress Tolerance. *Methods in Molecular Biology*, *1631*, 41–68. https://doi.org/10.1007/978-1-4939-7136-7_3

Staiger, D., & Brown, J. W. S. (2013). Alternative Splicing at the Intersection of Biological Timing, Development, and Stress Responses. *The Plant Cell*, *25*(10), 3640–3656. https://doi.org/10.1105/TPC.113.113803

Steinmann, R., & Dersch, P. (2013). Thermosensing to Adjust Bacterial Virulence in a Fluctuating Environment. *Future Microbiology*, *8*(1), 85–105. https://doi.org/10.2217/FMB.12.129

Steward, R. A., de Jong, M. A., Oostra, V., & Wheat, C. W. (2022). Alternative splicing in seasonal plasticity and the potential for adaptation to environmental change. *Nature Communications 2022 13:1*, *13*(1), 1–12. https://doi.org/10.1038/s41467-022-28306-8

Sun, L., Xu, Y., Bai, S., Bai, X., Zhu, H., Dong, H., … Song, C. P. (2019). Transcriptome-wide analysis of pseudouridylation of mRNA and non-coding RNAs in Arabidopsis. *Journal of Experimental Botany*, *70*(19), 5089–5600. https://doi.org/10.1093/JXB/ERZ273

Sun, Z., Hu, Y., Zhou, Y., Jiang, N., Hu, S., Li, L., & Li, T. (2022). tRNA-derived fragments from wheat are potentially involved in susceptibility to Fusarium head blight. *BMC Plant Biology*, *22*(1), 1–17. https://doi.org/10.1186/S12870-021-03393-9/FIGURES/7

Szabo, L., Morey, R., Palpant, N. J., Wang, P. L., Afari, N., Jiang, C., … Salzman, J. (2015). Statistically based splicing detection reveals neural enrichment and tissue-specific induction of circular RNA during human fetal development. *Genome Biology*, *16*(1), 1–26. https://doi.org/10.1186/S13059-015-0690-5/FIGURES/7

913 Ter Horst, A. M., Nigg, J. C., Dekker, F. M., Falk, B. W., & Pfeiffer, J. K. (2019). Endogenous Viral Elements
914 Are Widespread in Arthropod Genomes and Commonly Give Rise to PIWI-Interacting RNAs. *Journal*
915 *of Virology*, *93*(6), e02124-18. https://doi.org/10.1128/JVI.02124-18
916 Toomer, G., Gan, H., & Sztuba-Solinska, J. (2020). Long Non-coding RNAs Diversity in Form and Function:
917 From Microbes to Humans. *RNA Technologies*, *11*, 1–57. https://doi.org/10.1007/978-3-030-
918 44743-4_1
919 Tosar, J. P., & Cayota, A. (2020). Extracellular tRNAs and tRNA-derived fragments. *RNA Biology*, *17*(8),
920 1149–1167. https://doi.org/10.1080/15476286.2020.1729584
921 Tudek, A., Krawczyk, P. S., Mroczek, S., Tomecki, R., Turtola, M., Matylla-Kulińska, K., … Dziembowski, A.
922 (2021). Global view on the metabolism of RNA poly(A) tails in yeast Saccharomyces cerevisiae.
923 *Nature Communications 2021 12:1*, *12*(1), 1–14. https://doi.org/10.1038/s41467-021-25251-w
924 Twittenhoff, C., Heroven, A. K., Mühlen, S., Dersch, P., & Narberhaus, F. (2020). An RNA thermometer
925 dictates production of a secreted bacterial toxin. *PLOS Pathogens*, *16*(1), e1008184.
926 https://doi.org/10.1371/JOURNAL.PPAT.1008184
927 Verta, J. P., & Jacobs, A. (2022). The role of alternative splicing in adaptation and evolution. *Trends in*
928 *Ecology and Evolution*, *37*(4), 299–308. https://doi.org/10.1016/j.tree.2021.11.010
929 Verwilt, J., & Vromman, M. (2024). Current Understandings and Open Hypotheses on Extracellular
930 Circular RNAs. *Wiley Interdisciplinary Reviews: RNA*, *15*(6), e1872.
931 https://doi.org/10.1002/WRNA.1872
932 Wang, M., Weiberg, A., Dellota, E., Yamane, D., & Jin, H. (2017). Botrytis small RNA Bc-siR37 suppresses
933 plant defense genes by cross-kingdom RNAi. *RNA Biology*, *14*(4), 421–428.
934 https://doi.org/10.1080/15476286.2017.1291112
935 Wang, Y. J., Yang, B., Lai, Q., Shi, J. F., Peng, J. Y., Zhang, Y., … Yin, D. (2021). Reprogramming of m6A
936 epitranscriptome is crucial for shaping of transcriptome and proteome in response to hypoxia. *RNA*
937 *Biology*, *18*(1), 131–143. https://doi.org/10.1080/15476286.2020.1804697
938 Wang, Z., Sun, J., Zu, X., Gong, J., Deng, H., Hang, R., … Cao, X. (2022). Pseudouridylation of chloroplast
939 ribosomal RNA contributes to low temperature acclimation in rice. *New Phytologist*, *236*(5), 1708–
940 1720. https://doi.org/10.1111/NPH.18479
941 Weiberg, A., Bellinger, M., & Jin, H. (2015). Conversations between kingdoms: small RNAs. *Current*
942 *Opinion in Biotechnology*, *32*, 207–215. https://doi.org/10.1016/J.COPBIO.2014.12.025
943 Weiberg, A., Wang, M., Bellinger, M., & Jin, H. (2014). Small RNAs: A new paradigm in plant-microbe
944 interactions. *Annual Review of Phytopathology*, *52*(Volume 52, 2014), 495–516.
945 https://doi.org/10.1146/ANNUREV-PHYTO-102313-045933/CITE/REFWORKS
946 Wilson, T. J., & Lilley, D. M. J. (2021). The potential versatility of RNA catalysis. *Wiley Interdisciplinary*
947 *Reviews: RNA*, *12*(5), e1651. https://doi.org/10.1002/WRNA.1651
948 Wood, S. A., Biessy, L., Latchford, J. L., Zaiko, A., von Ammon, U., Audrezet, F., … Pochon, X. (2020).
949 Release and degradation of environmental DNA and RNA in a marine system. *Science of The Total*
950 *Environment*, *704*, 135314. https://doi.org/10.1016/J.SCITOTENV.2019.135314
951 Wu, X., Wang, J., Wu, X., Hong, Y., & Li, Q. Q. (2020). Heat Shock Responsive Gene Expression Modulated
952 by mRNA Poly(A) Tail Length. *Frontiers in Plant Science*, *11*, 555049.
953 https://doi.org/10.3389/FPLS.2020.01255/BIBTEX
954 Xiao, Y. L., Liu, S., Ge, R., Wu, Y., He, C., Chen, M., & Tang, W. (2023). Transcriptome-wide profiling and
955 quantification of N6-methyladenosine by enzyme-assisted adenosine deamination. *Nature*

Biotechnology, *41*(7), 993. https://doi.org/10.1038/S41587-022-01587-6

Xue, Z., Tian, W., Han, Y., Li, S., Guo, J., He, H., … Zhang, W. (2024). Environmental RNA metabarcoding for ballast water microbial diversity: Minimizing false positives. *Science of The Total Environment*, *955*, 176902. https://doi.org/10.1016/J.SCITOTENV.2024.176902

Yablonovitch, A. L., Deng, P., Jacobson, D., & Li, J. B. (2017). The evolution and adaptation of A-to-I RNA editing. *PLOS Genetics*, *13*(11), e1007064. https://doi.org/10.1371/JOURNAL.PGEN.1007064

Yan, Y. W., Zou, B., Zhu, T., Hozzein, W. N., & Quan, Z. X. (2017). Modified RNA-seq method for microbial community and diversity analysis using rRNA in different types of environmental samples. *PLOS ONE*, *12*(10), e0186161. https://doi.org/10.1371/JOURNAL.PONE.0186161

Yang, H., Cui, Y., Feng, Y., Hu, Y., Liu, L., & Duan, L. (2023). Long Non-Coding RNAs of Plants in Response to Abiotic Stresses and Their Regulating Roles in Promoting Environmental Adaption. *Cells*, *12*(5), 729. https://doi.org/10.3390/CELLS12050729/S1

Yang, X., Liu, Y., Zhang, H., Wang, J., Zinta, G., Xie, S., … Nie, W. F. (2020). Genome-Wide Identification of Circular RNAs in Response to Low-Temperature Stress in Tomato Leaves. *Frontiers in Genetics*, *11*, 591806. https://doi.org/10.3389/FGENE.2020.591806/BIBTEX

Yates, M. C., Furlan, E., Thalinger, B., Yamanaka, H., & Bernatchez, L. (2023). Beyond species detection—leveraging environmental DNA and environmental RNA to push beyond presence/absence applications. *Environmental DNA*, *5*(5), 829–835. https://doi.org/10.1002/EDN3.459

Yates, Matthew C., Derry, A. M., & Cristescu, M. E. (2021). Environmental RNA: A Revolution in Ecological Resolution? *Trends in Ecology and Evolution*, *36*(7), 601–609. https://doi.org/10.1016/j.tree.2021.03.001

Ye, C., Long, Y., Ji, G., Li, Q. Q., & Wu, X. (2018). APAtrap: identification and quantification of alternative polyadenylation sites from RNA-seq data. *Bioinformatics*, *34*(11), 1841–1849. https://doi.org/10.1093/BIOINFORMATICS/BTY029

Yu, S., & Kim, V. N. (2020). A tale of non-canonical tails: gene regulation by post-transcriptional RNA tailing. *Nature Reviews Molecular Cell Biology 2020 21:9*, *21*(9), 542–556. https://doi.org/10.1038/s41580-020-0246-8

Yue, Y., Liu, J., & He, C. (2015). RNA N6-methyladenosine methylation in post-transcriptional gene expression regulation. *Genes & Development*, *29*(13), 1343–1355. https://doi.org/10.1101/GAD.262766.115

Zaringhalam, M., & Papavasiliou, F. N. (2016). Pseudouridylation meets next-generation sequencing. *Methods*, *107*, 63–72. https://doi.org/10.1016/J.YMETH.2016.03.001

Zhang, A., Jiang, X., Zhang, F., Wang, T., & Zhang, X. (2019). Dynamic response of RNA editing to temperature in grape by RNA deep sequencing. *Functional & Integrative Genomics*, *20*(3), 421. https://doi.org/10.1007/S10142-019-00727-7

Zhang, C. (2008). MicroRNomics: A newly emerging approach for disease biology. *Physiological Genomics*, *33*(2), 139–147. https://doi.org/10.1152/PHYSIOLGENOMICS.00034.2008/ASSET/IMAGES/LARGE/ZH700608321400 02.JPEG

Zhang, J., Li, S., Li, L., Li, M., Guo, C., Yao, J., & Mi, S. (2015). Exosome and Exosomal MicroRNA: Trafficking, Sorting, and Function. *Genomics, Proteomics & Bioinformatics*, *13*(1), 17–24. https://doi.org/10.1016/J.GPB.2015.02.001

Zhang, X. O., Dong, R., Zhang, Y., Zhang, J. L., Luo, Z., Zhang, J., … Yang, L. (2016). Diverse alternative

back-splicing and alternative splicing landscape of circular RNAs. *Genome Research*, *26*(9), 1277–1287. https://doi.org/10.1101/GR.202895.115

Zhao, B., Deng, J., Ma, M., Li, N., Zhou, J., Li, X., & Luan, T. (2024). Environmentally relevant concentrations of 2,3,7,8-TCDD induced inhibition of multicellular alternative splicing and transcriptional dysregulation. *Science of The Total Environment*, *919*, 170892. https://doi.org/10.1016/J.SCITOTENV.2024.170892

Zhao, B. S., & He, C. (2014). Pseudouridine in a new era of RNA modifications. *Cell Research 2015 25:2*, *25*(2), 153–154. https://doi.org/10.1038/cr.2014.143

Zhao, X. F., Liang, L. Q., Liew, H. J., Chang, Y. M., Sun, B., Wang, S. Y., … Zhang, L. M. (2021). Identification and Analysis of Long Non-coding RNAs in Leuciscus waleckii Adapted to Highly Alkaline Conditions. *Frontiers in Physiology*, *12*, 665268. https://doi.org/10.3389/FPHYS.2021.665268/BIBTEX

Zhou, J., & Li, Q. Q. (2023). Stress responses of plants through transcriptome plasticity by mRNA alternative polyadenylation. *Molecular Horticulture*, *3*(1), 1–14. https://doi.org/10.1186/S43897-023-00066-Z/FIGURES/2

Table 1. Summary of eRNA types beyond mRNA with potential applications in eRNA studies. Table highlights their functions, significance for eRNA research, and current limitations in application. Also, it outlines the most common detection techniques and suggests improvements to make these methods more suitable for eRNA studies in diverse and complex environmental contexts. Finally, we give our subjective estimation of the current overall feasibility to incorporate the respective RNA type into eRNA studies in the upcoming 5-10 years (*** - high, ** - intermediate, * - low).

Molecule	Function	Significance for eRNA research	Limitation	Detection techniques	Required technical improvements	Current feasibility or eRNA research
rRNA	Form ribosome's core, facilitates protein synthesis	suitable for taxonomic and activity profiling, more stable and abundant than mRNA	Limited resolution for closely related species, focuses on translational potential	rRNA-seq, qPCR, metatranscriptomics	Develop tools for higher taxonomic resolution and integrate functional analysis	***
tRNA	Transfer amino acids during translation, regulates translational machinery	more stable and abundant than mRNA, tRNA modifications occurring in extreme environments	poorly annotated modifications, low sequence variability	tRNA-seq, Northern blotting, qPCR	Improve annotations and study tRNA modifications in diverse environments	*
Ribozymes	Catalyze biochemical reactions independently of proteins	potential makers of adaption	low abundance, requires targeted biochemical validation	Directed evolution assays, RNA-seq, in vitro validation	Improve sensitivity of detection methods	**
circRNAs	Act as miRNA sponge, regulates proteins, stable under stress	Highly stable, persists longer in environmental samples	Limited understanding in non-model organisms, low abundance	CircRNA-seq, RIP	Optimize detection for low-abundance circRNAs	**
RNA thermosensors	Respond to temperature via conformational changes	Potential markers of temperature stress in species communities	Resource-intensive detection, mainly studied in bacteria, structural analysis required	SHAPE-seq, mutational assays	Enable conformation analyses from environnemental samples	*
sncRNAs	Regulate genes post-transcriptionally	Potential markers of stress response, microbial community interactions and inter-organismal communication	Low abundance, low sequence variability due to size, limited annotation for non-model species	Adjusted small RNA-seq	Improve annotation tools, expand genetic reference databases	**(*)
lncRNAs	Control transcription, remodels chromatin, aids adaptation	Potential markers of stress response, and host-pathogen interactions	Poorly conserved, requires extensive sequencing, functions poorly understood in non-model organisms	RNA-seq, ChIP	Expand functional and genetic reference databases of non-model organisms	*
tRFs	Modulate translation and stress responses	Potential markers of stress response	Challenging detection, small size hinders taxonomic annotation, functions poorly characterized	tRNA-seq, small RNA-seq	Expand functional understanding, improve sensitivity of sequencing techniques	*

Table 2. Summary of eRNA processes with potential applications in eRNA studies discussed in this manuscript. Table highlights their functions, significance for eRNA research, and current limitations in application. Also, it outlines the most common detection techniques and suggests improvements to make these methods more suitable for eRNA studies in diverse and complex environmental contexts. Finally, we give our subjective estimation of the current feasibility to incorporate the respective RNA type into eRNA studies in the upcoming 5-10 years (*** - high, ** - intermediate, * - low).

RNA process	Function	Significance for eRNA research	Limitation	Detection techniques	Required technical improvements	Current feasibility or eRNA research
RNA editing	Alters RNA sequences post-transcriptionally for adaptation	Indicates adaptive molecular changes and stress responses; host-pathogen dynamics	Requires intensive sequencing and computational resources	RNA-seq, editing-specific tools	Enhance RNA editing detection bioinformatics	**
RNA methylation	Regulates RNA stability and translation via chemical modifications	Indicates cellular stress responses, metabolic activity, and developmental states	Costly, requires specialized tools like mass spectrometry	LC-MS/MS, nanopore, MeRIP-Seq	Simplify and lower cost of modification-specific sequencing	***
alternative splicing	Diversifies gene expression through alternative splicing	Enables organismal plasticity and adaptive responses to environmental pressures	Sequencing resource-demanding, limited functional annotations	High-depth RNA-seq, splice-aware aligners	Increase accessibility to splice-aware alignment tools	**
RNA pseudo-uridylation	Stabilizes RNA and influences folding and interactions via chemical modifications	Indicator of organisms' resilience to extreme conditions	Detection is resource-intensive, functions often unknown in non-model species	LC-MS/MS, specialized RNA-seq platforms	Enhance detection techniques for low-abundance modifications	*
Polyadenylation and RNA tailing	Modifies RNA stability and degradation via nucleotide additions to 3' ends	Affecting RNA persistence in organisms and in the environment	Highly variable and transient, requires specific tools for accurate analysis	Poly(A)-tail-seq, nanopore-seq	Refine sequencing platforms for transient and variable RNA tails	*

Table 3. Regulatory effects of RNA processes on RNA types and their regulatory effects.

RNA process	Affected RNA types	Regulatory effects
RNA editing	lncRNAs, sncRNAs (e.g., miRNAs), piRNAs, ribozymes, RNA vsiRNAs	Alters sequence composition, target specificity, and function
RNA methylation (e.g., m6A)	mRNAs, lncRNAs, tRNAs, circRNAs, sncRNAs, tRFs	Regulates RNA stability, degradation, export, and translation efficiency
Alternative splicing	mRNAs, lncRNAs, circRNAs	Creates alternative isoforms
RNA pseudouridylation	mRNA, rRNAs, tRNAs, sncRNAs	affects stability, folding, and functionality
Polyadenylation and RNA tailing	mRNAs, lncRNAs, vsiRNAs	Regulates transcript lifespan and translational potential; tags RNA for degradation or protection

Box 1 Summary of the key research questions that could be addressed using alternative RNA types or RNA processes beyond conventional analysis of mRNA, along with the current main challenges in analyzing these and potential solutions.

Key questions for alternative eRNAs	Main challenges	Potential solutions
• Response to environmental stressors • Adaptation • Species interactions • eRNA dynamics • Physiological condition of organisms • Community assemblages	• Need of high sequencing depth • Expensive specialized sequencing techniques • Taxonomic assignment of very conserved and/or short RNA fragments • Lack of genetic reference data • Degradation in the environment obscuring real biological patterns • Low abundance of individual RNA types	• Sequencing costs continue to decline • Library preparation, enrichment and sequencing methods are being developed at an unprecedented speed with more efficient workflows evolving continuously • Species-level assignment might not be necessary for many biological inferences. Assignment to higher taxonomic level may be sufficient if the associated function is very conserved. • Genetic data, including RNA-seq data continues to be produced at a fast speed for a growing list of species, which can all serve as future genetic reference data • New enrichment methods continue to be developed, enabling access to more low-abundant RNA types