Environmental transcriptomics under heat stress: Can environmental RNA reveal changes in gene expression of aquatic organisms?

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Author contributions:
RMH and MEC designed the study. RMH and MCY conducted the experiment. RMH conducted all molecular work. RMH and FJJC performed the bioinformatic analysis and produced the figures. RMH wrote the first draft of the manuscript and all authors contributed to editing the manuscript.

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Abstract: To safeguard biodiversity in a changing climate, we require taxonomic information about species turnover and insights into the health of organisms. Environmental DNA approaches are increasingly used for species identification, but cannot provide functional insights. Transcriptomic methods reveal the physiological states of macroorganisms, but are currently species specific and require tissue sampling or animal sacrifice, making community-wide assessments challenging. Here, we test if broad functional information (activity level of the transcribed genes) can be harnessed from environmental RNA (eRNA), which includes extra-organismal RNA from macroorganisms along with whole microorganisms. We exposed *Daphnia pulex* as well as phytoplankton prey and microorganism colonizers to control (20 °C) and heat stress (28 °C) conditions for seven days. We sequenced eRNA from tank water (after complete removal of *Daphnia*) as well as RNA from *Daphnia* tissue, enabling comparisons of extra-organismal and organismal RNA based gene expression profiles. Both RNA types detected similar gene expression responses. Using eRNA, we identified 42 *Daphnia* genes to be differentially expressed following heat stress. Of these, 62% were also differentially expressed and exhibited similar levels of relative expression in organismal RNA. Both RNA types recovered similar *Daphnia* heat stress responses via gene ontology terms. In addition to the extra-organismal *Daphnia* response, eRNA detected community-wide heat stress responses consisting of 199 differentially expressed genes across 9 taxa, and distinct functional profiles via KEGG orthologs. Our study demonstrates that environmental transcriptomics based on eRNA can non-invasively reveal gene expression responses of macroorganisms following environmental changes, with broad potential implications for the biomonitoring of ecological health across the trophic chain.

Introduction

In an era of ecological crises, monitoring the presence and health of organisms within an ecosystem is essential for the preservation of biodiversity. Environmental DNA is routinely used for biomonitoring, but such applications are limited to species detection [1,2]. The RNA molecule provides an additional layer of functional information, as transcribed genes reflect an organism's physiological status [3]. Thus, transcriptomics is used to infer the ecological health of organisms, but such surveys are species-specific and dependent on sampling organismal RNA (oRNA) directly, which is labor-intensive and requires tissue collection or animal sacrifice [4–9]. Non-invasive environmental RNA (eRNA) approaches would overcome these limitations by providing insights into the health of populations and communities [10–13]. However, using eRNA to inform on ecological health only been theorized (see [13]) and remains yet to be empirically tested.

We broadly define eRNA as RNA extracted from the environment, including extra-organismal RNA released by macroorganisms as well as whole microorganisms. Microbiologists have long extracted RNA from bulk samples to profile gene expression in microbial communities, referred to as metatranscriptomics [14–16]. Despite more than two decades of research, metatranscriptomics remains limited to studying the gene expression of microorganisms captured in bulk samples rich in oRNA. The general assumption has been that the eRNA released by macroorganisms into the environment is labile and degrades too rapidly to be reliably detected or quantified (but see [11]). However, recent work has demonstrated that macroorganisms do shed and/or excrete RNA into their surrounding environment and that this eRNA can be successfully extracted and quantified [17–21]. Despite this evidence supporting the

78 extractability of eRNA, environmental transcriptomics, defined here as eRNA based gene
79 expression profiling, remained untested.

80 In aquatic systems, environmental conditions modulate biodiversity and are reflected in
81 changes of metatranscriptomes [14,22–24]. Temperature is a key abiotic factor that influences
82 the physiology and fitness of ectotherms due to the relationship between body and environmental
83 temperatures [25–27]. Rapid increases in temperature, such as heatwaves, can exceed an aquatic
84 ectotherms thermal limits and exponentially increase its standard metabolic rate, resulting in
85 decreases in fitness and survival [26–29]. Transcriptomics is widely used to study the heat stress
86 response of many taxa, including fish [7,30–33], coral holobionts [34–36], molluscs [6,37], and
87 copepods [38,39]. *Daphnia pulex*, a key bioindicator species, exhibit widespread downregulation
88 of genes involved in cellular and metabolic processes under heat stress [40,41]. Clearly, gene
89 expression surveys can provide species-specific functional insights important for conserving
90 aquatic biodiversity [42–44]. However, a species by species analysis based on oRNA cannot
91 capture community-wide changes without extensive efforts. Mapping eRNA reads to the
92 reference genomes of multiple target species and using existing metatranscriptomic pipelines,
93 could enable environmental transcriptomics to revolutionize biological monitoring by revealing
94 gene expression responses across the trophic chain.

95 We constructed simple mock freshwater communities containing *Daphnia pulex,* as well
96 as three phytoplankton species and opportunistic microorganisms that colonized the artificial
97 lake water. The communities were exposed to control (20 °C) and near-lethal *Daphnia* heat
98 stress (28 °C) conditions for seven days. To enable comparisons between eRNA and oRNA
99 based gene expression profiles, we sequenced eRNA from tank water (after complete *Daphnia*
100 removal) and oRNA from *Daphnia* tissue. We conducted differential gene expression analyses

101 between the control and heat stress conditions and compared the *Daphnia* functional stress
102 response identified in eRNA and oRNA. In addition to the *Daphnia* analysis, we used eRNA to
103 investigate the community-wide heat stress response. We hypothesized that 1) eRNA contains a
104 subset of the differentially expressed *Daphnia* genes observed in oRNA after exposure to heat
105 stress and 2) eRNA captures transcriptional responses to heat stress. Based on our findings, we
106 discuss the potential of eRNA based transcriptomics for the biological monitoring of ecological
107 communities and also provide recommendations to overcome potential limitations.
108

109 **Results**

110 Daphnia pulex *gene detection and expression profiles*

111 Since we pre-filtered tank water at 60 µm to remove *Daphnia*, all *Daphnia* reads within
112 environmental RNA (eRNA) samples are of extra-organismal origin. Sequencing of eRNA from
113 tank water and organismal RNA (oRNA) from *Daphnia* tissue detected 3,941 (21%) and 17,586
114 (95%) genes (in at least 3 of 4 replicates), respectively, of the 18,449 genes that compose the
115 *Daphnia pulex* reference genome [45]. All *Daphnia* genes detected in eRNA were also detected
116 in oRNA. A principal component analysis of the regularized log normalized expression for the
117 300 most variant genes showed separation between experimental groups (Fig. 1). The samples
118 were separated across the first principal component (PC1) by RNA type and across the second
119 principal component (PC2) by temperature, clustering eRNA, oRNA, 20 °C and 28 °C within
120 their respective groups. The only exception was 20 °C eRNA sample T1, which unexpectedly
121 clustered closely with the 28 °C eRNA group. PC1 and PC2 accounted for 63% of explained
122 variance of all principal components (Fig. S1).

123

124 Daphnia pulex *differential gene expression*

125 Comparison of gene expression profiles at 20 °C and 28 °C identified 42 significantly
126 differentially expressed genes (DEG; FDR-adjusted p-value <0.1) from eRNA (8 upregulated
127 and 34 downregulated) and 3108 DEGs from oRNA samples (1470 upregulated and 1638
128 downregulated). All eRNA significant DEGs were highly up and down regulated (exceeding
129 Log_2 2 fold change), as well as 46 and 226 oRNA DEGs, respectively. Of the 93% of eRNA
130 DEGs that exhibited the same directional change (up/down regulated) as in oRNA (Table S1),
131 62% (5 upregulated and 21 downregulated) were also differentially expressed in oRNA (Fig.
132 S2). A heatmap comparing the relative expression (Z-score) of the 26 *D. pulex* DEGs common to
133 eRNA and oRNA revealed four distinct clusters based on hierarchical clustering analyses,
134 separating samples and genes by temperature and directional change, respectively (Fig. 2).
135 Across both temperature conditions, eRNA and oRNA clustered together and were not separated
136 into RNA types, indicating that eRNA and oRNA exhibit similar levels of relative gene
137 expression for these DEGs. The only exception was eRNA 20 °C T1 sample which, similar to
138 the PCA, unexpectedly clustered with the 28 °C samples.

139

140 Daphnia pulex *functional annotation*

141 Gene ontology (GO) enrichment analysis of eRNA detected genes identified 57 GO terms
142 as enriched (FDR-corrected p-value <0.05), with 33, 6, and 18 belonging to Biological Processes
143 (BP), Cellular Component (CC) and Molecular Function (MF) domains, respectively (Figs. 3 and
144 S3). Of the 42 significant *D. pulex* DEGs identified from eRNA, 27 had GO annotations from the
145 reference genome [45] (Fig. S4-5). GO enrichment analysis of eRNA DEGs identified chitin

146 metabolic process as enriched (FDR-corrected p-value = 0.016) between expected and observed
147 counts (Fig. 3).
148 GO enrichment analysis of oRNA detected genes identified 119 GO terms as enriched
149 (FDR-corrected p-value <0.05), with 100, 1, and 18 belonging to BP, CC and MF domains,
150 respectively (Figs. 3 and S3). Of the 3108 DEGs identified from oRNA, 2259 had GO
151 annotations from the reference genome [45] (Figs. S4-5). GO enrichment analysis of oRNA
152 DEGs identified 17 GO terms as enriched (FDR-corrected p-value <0.05), including chitin
153 metabolic process, proteolysis, negative regulation of hydrolase activity, negative regulation of
154 proteolysis and response to stimulus (Fig S3). Of the 26 genes found to be commonly DE in both
155 eRNA and oRNA, the GO terms chitin metabolic process and chitin binding were enriched
156 (FDR-corrected p-value <0.05; Fig. 3).
157
158 *Community composition, differential gene expression and functional annotation*
159 eRNA reads originated from a simple mock freshwater community that we constructed,
160 consisting of *Daphnia pulex* as well as three phytoplankton species and opportunistic
161 microorganisms that colonized the artificial lake water. All non-*Daphnia* eRNA reads likely
162 originate from whole microorganisms that were captured on the filter. We used the SqueezeMeta
163 pipeline [46] to align eRNA reads to the GenBank nr database using its LCA algorithm, which
164 identified 434 taxa. The community composition and relative abundances remained similar
165 between 20 °C and 28 °C conditions (Fig. S6). Across eRNA samples, an average of 38% of all
166 reads were aligned to nine species including four eukaryotes that were a priori known to persist
167 in the communities and five highly abundant taxa identified by the SqueezeMeta analysis (Table
168 S2). We detected 10,245 genes from these nine species, with the majority originating from *D.*

169 *pulex, Scenedesmus quadricauda and Ankistrodesmus falcatus,* with 3941, 3223 and 2123 genes,
170 respectively (Table S3). Differential gene expression analysis between 20 °C and 28 °C eRNA
171 samples was performed for each of the nine species and identified a total of 199 significantly
172 differentially expressed genes (Table S3). The most DEGs were identified for *A. falcatus*, *S.*
173 *quadricauda* and *D. pulex*, with 72, 47 and 42 DEGs respectively.
174 Within all eRNA reads, 12,210 KEGG IDs were identified and a non-metric
175 multidimensional scaling plot of these KEGG IDs revealed distinct functional profiles between
176 20 °C and 28 °C communities (Fig. S7). Of all KEGG IDs, 127 were identified as significantly
177 differentially expressed (DE; false discovery rate-adjusted p-value <0.1) between 20 °C and 28
178 °C (Table S4). Many of these DE KEGG IDs correspond to proteins and pathways involved in
179 stress responses, including metabolic pathway, p53 signaling pathway, ubiquitin mediated
180 proteolysis and cyclin-dependent kinase 4 (Table S4).
181
182 **Discussion**
183 The use of environmental RNA (eRNA) based transcriptomics remained previously
184 untested despite recent evidence demonstrating that extra-organismal eRNA persists in the
185 environment long enough to be extracted [17–21]. Our findings revealed that environmental
186 transcriptomics is sensitive in detecting transcriptional heat stress responses even without
187 sampling source organisms directly. From eRNA released by *Daphnia pulex* into the tank water,
188 we detected thousands of *Daphnia* genes and identified a subset of heat stress relevant genes to
189 be differentially expressed (DE) between the two temperature conditions, with levels of relative
190 expression similar to organismal RNA (oRNA). We also detected community wide changes in
191 functional profiles. Our study demonstrates the potential of environmental transcriptomics to

192 reveal the functional response of biotic communities to environmental stress, with broad
193 implications for biomonitoring ecological health across the trophic chain.
194
195 *Recovery of* Daphnia pulex *extra-organismal eRNA*
196 Several studies have recently demonstrated robust detection and persistence of extra-
197 organismal eRNA in the environment [17,19,21]. Our results are consistent with these findings
198 as we recovered substantial quantities of *Daphnia* eRNA, demonstrated by an average of 0.51%
199 of all reads mapping to the *D. pulex* reference genome (Table S2). This relatively high recovery
200 of *Daphnia* reads was particularly notable, given the general expectation that within
201 environmental samples, potentially low-abundance eRNA is 'competing' to be sequenced with
202 larger quantities of oRNA from whole microorganisms. This encouraging finding suggests that
203 most of the eRNA captured is in a protected state (e.g., cellular, vesicular, or in stable molecular
204 complexes) while free RNA forms, prone to degradation, are very short lived and potentially
205 undetectable. Our recovery of extra-organismal eRNA reads (0.51%) was similar to
206 metagenomic studies of natural aquatic ecosystems that found between 0.27% and 1.25% of
207 environmental DNA (eDNA) reads to be likely of extra-organismal origin [47–50].
208
209 Daphnia pulex *extra-organismal eRNA differential gene expression analysis*
210 Our broad environmental transcriptomics approach was able to detect 3,941 *D. pulex*
211 genes from eRNA, building upon targeted RT-PCR of eRNA which detected 6 fish genes [20].
212 Of the genes we detected, 42 were found to be significantly differentially expressed genes
213 (DEGs) under temperature stress. As expected, we identified many more (3,108) DEGs from
214 *Daphnia* oRNA. This discrepancy in DEG identification can be easily attributed to large

215 differences in the number of reads that mapped to the reference genome. On average, 1 and 200
216 million reads mapped to the *D. pulex* reference genome using eRNA and oRNA, respectively
217 (Table S2). Having less than 1% of eRNA reads matching *D. pulex* limited our power to detect
218 DEGs, but is unsurprising given that the libraries were generated from diverse environmental
219 samples which included RNA transcripts from over 400 taxa, whereas the oRNA libraries were
220 prepared from concentrated *D. pulex* tissue samples. On the other hand, we believe this diversity
221 of eRNA within water samples to be beneficial as it opens up exciting possibilities of conducting
222 community-wide surveys that encompass macro- and micro-organisms across the trophic chain.
223 For our proof-of-concept study, we performed deep sequencing (average of 158 million reads per
224 eRNA sample, Table S2) in anticipation of such diverse eRNA samples, enabling sufficient
225 recovery of *D. pulex* eRNA for gene expression analysis. Despite the lower abundance of *D.*
226 *pulex* reads from eRNA than oRNA, 93% of eRNA DEGs exhibited the same directional change
227 (up/down regulated) as in oRNA (Table S1). Additionally, the majority of eRNA DEGs (62%)
228 were also differentially expressed in oRNA and these commonly shared DEGs exhibited similar
229 levels of relative expression in both RNA types (Fig 2). These results demonstrate that similar to
230 oRNA, our environmental transcriptomics approach yielded adequate sensitivity in detecting
231 changes in gene expression of a source organism in response to heat stress.
232 Changes in gene expression are often observed before phenotypic and demographic
233 effects occur and may therefore act as an early indication of ecological stress [51–53]. We found
234 that eRNA and oRNA recovered similar *D. pulex* chronic heat stress responses which consisted
235 of extensive downregulation of genes associated with cellular and metabolic processes,
236 membrane, extracellular region, collagen and cuticle structure (Figs. 3 and S4). These results are
237 consistent with studies that also found chronically heat stressed *Daphnia* to exhibit widespread

238 downregulation of genes with similar functions [40,41]. This widespread downregulation of
239 genes has been hypothesized to be a molecular compensatory mechanism for *Daphnia* at near-
240 lethal temperatures to sacrifice their long-term fitness for immediate survival [41].

241 After seven days of heat exposure, we found few heat shock genes (4 of 67) to be
242 significantly differentially expressed but only with slight upregulation (log_2 fold change of 1.1-
243 1.3; Table S5), from *Daphnia* oRNA. This corroborates findings that indicate heat shock genes
244 to be central for *Daphnia* in mitigating cell damage following acute (hours) heat exposure, but
245 less important for the chronic (days) heat stress response [40,41]. Multiple tropical marine fish
246 species similarly do not differentially express heat shock genes during chronic heat stress [31],
247 suggesting that heat shock genes might not be optimal for assessments of communities under
248 chronic heat stress. Furthermore, we detected chitin related gene ontology (GO) terms to be
249 commonly enriched in both eRNA and oRNA DEGs (Fig. 3), corroborating *Daphnia* studies that
250 linked chitin genes to environmental stressors [40,54–56]. Hydrolytic and proteolytic related GO
251 terms were enriched in eRNA (Fig. 3), and are commonly involved in *Daphnia* and copepod heat
252 stress response by stabilizing and cleaning up misfolded proteins [38,40]. It is promising that
253 using eRNA, we were able to detect thousands of genes and identify a subset as differentially
254 expressed with similar levels of relative expression as oRNA, and detect the widespread
255 downregulation of genes associated with chronic heat stress. We also found that many GO terms
256 were commonly shared in both RNA types, and that stress related terms were enriched in eRNA.
257 Collectively, our results indicate that environmental transcriptomics based on eRNA is sensitive
258 in detecting heat stress responses of source organisms through changes in gene expression and
259 functional GO terms. Future empirical work is necessary to determine how our results extend to
260 more complex natural ecosystems.

261

262 *Community composition and function*

263 Since we pre-filtered the tank water at 60 μm to remove *Daphnia*, all non-*Daphnia* RNA
264 recovered in our eRNA samples likely originates from whole microorganisms that passed the
265 filter. After mapping all eRNA reads to GenBank nr, we found the community composition to
266 remain largely unchanged for 20 °C and 28 °C conditions (Fig. S6). Within our eRNA samples,
267 gene expression analysis of nine species (across five phyla) detected 10,245 genes with 199 of
268 these being significantly differentially expressed (DE) between 20 °C and 28 °C (Table S3).
269 Although community compositions remained similar, analysis of KEGG orthologs revealed
270 distinct functional profiles for communities in the two temperature treatments (Fig. S7). We
271 found many stress-related KEGG orthologs to be DE, including proteins involved in binding,
272 kinase oxidoreductase, and cellular senescence, a pathway indicative of cellular arrest and DNA
273 damage (Table S4). Our community wide results reflect similar patterns of differential gene
274 expression and shifts in functional terms observed in conventional aquatic metatranscriptomic
275 studies based on bulk microorganism samples [14–16,22,57–60]. For example, a freshwater
276 metatranscriptome likely under heat stress exhibited similar stress responses which included
277 oxidoreductase, binding and kinase proteins [61]. We build upon this rich metatranscriptomics
278 literature by demonstrating that in addition to yielding microorganism insights, environmental
279 transcriptomics can also non-invasively detect the gene expression response of macroorganisms
280 to environmental stress.

281

282 *Recommendations and future directions*

283 Interest in non-lethal transcriptomics is growing, as such an approach increases animal
284 welfare, allows for repeated survey of individuals, and linking gene expression to fitness
285 following exposure to stress [8,62,63]. However, current non-lethal methods are species specific
286 and involve animal handling, and sampling for blood or tissue, which may still result in organism
287 stress and reduced survival upon release into the environment [64–66]. Inferring the
288 physiological state of organisms from non-invasive eRNA samples would represent a substantial
289 improvement for animal welfare as we could circumvent these stressful sampling procedures.
290 We demonstrated that environmental transcriptomics based on eRNA can non-invasively detect
291 the heat stress response of progenitor organisms, with similar sensitivity to that of the
292 conventional and invasive oRNA approach.

293 Perhaps the most exciting prospect of environmental transcriptomics is the potential to
294 monitor the ecological health of entire communities and thereby gain insights into ecosystem
295 level conditions. Biomonitoring traditionally relies upon identifying bioindicator taxa that are
296 correlated with environmental conditions, but transcriptomic surveys can provide valuable early
297 warnings since gene expression changes are likely to occur before compositional shifts could be
298 detected [12]. Conventional metatranscriptomics has demonstrated that gene expression profiling
299 of microorganisms reflects environmental conditions [22,58,67,68]. Environmental
300 transcriptomics based on eRNA extends beyond microorganisms by revealing expression signals
301 from extra-organismal eRNA, thus providing functional information of species across the trophic
302 chain. Such an approach could enable researchers to correlate functional pathways expressed by
303 community assemblages to ecosystem level conditions [13]. Gaining insights into the ecological

304 health of populations and communities, as well as ecosystem level conditions, will enable
305 conservation initiatives to be proactive in their efforts and respond before local extinction events
306 occur.

307 We anticipate multiple challenges for environmental transcriptomic biomonitoring of
308 natural ecosystems, such as achieving adequate sequencing depth of eRNA in a cost-efficient
309 manner (Table 1). However, sequencing technologies are rapidly advancing and the costs
310 continue to decrease [69]. The continuation of such rapid technological advancement will enable
311 future studies to generate more reads less expensively, thereby increasing the sensitivity of
312 environmental transcriptomics to detect extra-organismal eRNA transcripts. Additionally, the
313 continued development of low input mRNA-seq library preparation kits will enable researchers
314 to prepare libraries for environmental transcriptomics with minimal template eRNA. Molecular
315 technological advancements that enhance extraction efficiency, enrich eukaryotic RNA and
316 remove ribosomal RNA from diverse samples will increase the yield of coding eRNA and thus
317 the power of environmental transcriptomics.

318 While sequencing and molecular advancements will resolve challenges associated with
319 eRNA depth, perhaps the largest remaining challenge for environmental transcriptomics to
320 overcome is the lack of a baseline to compare against. Differential gene expression analyses are
321 conducted by comparing a treatment (i.e. stressed community) against a control 'baseline' (i.e.
322 healthy community), to identify genes involved with the stress response. However, in natural
323 ecosystems, it is difficult to obtain a control baseline due to population demographics and
324 multiple stressors acting simultaneously on a system. This is similarly shared by
325 metatranscriptomics, and multiple statistical and mathematical modelling solutions have been
326 developed to overcome this problem [70–72]. Additionally, we propose supervised machine

327 learning (SML) models as a baseline-free solution for assessing community health using
328 environmental transcriptomics. SML models could be trained with existing datasets
329 (transcriptomics, metatranscriptomics and metagenomic) that have identified genes, functional
330 pathways and taxa associated with varying degrees of ecological quality [73]. Once trained,
331 environmental transcriptomic data could be used as input and the SML model would predict the
332 stressor most likely at play and the ecological health status of the community.

333 By providing functional insights in a non-invasive manner, eRNA based approaches
334 could push beyond the limitations of eDNA species detection and oRNA invasive transcriptomic
335 surveys. We demonstrated, for the first time, that eRNA can be used to detect thousands of
336 genes, and that environmental transcriptomics is sensitive enough to identify a subset of
337 functionally relevant genes as differentially expressed under heat stress, with relative expression
338 levels similar to the traditional oRNA approach. Additionally, within all eRNA reads, we were
339 able to reveal changes in functional profiles for the community in response to heat stress.
340 Collectively, these findings demonstrate the potential of environmental transcriptomics to
341 revolutionize biological monitoring by providing functional information across the trophic chain
342 and insights into the health of ecological communities. Our study represents a significant
343 advancement for the non-invasive biomonitoring of ecological community health, but future
344 empirical work is necessary to determine if environmental transcriptomics can be extended to
345 natural ecosystems.
346

347 **Materials and Methods**

348 *Experimental design*

349 We constructed simple mock freshwater communities composed of *Daphnia pulex,* three
350 algae species (*Ankistrodesmus falcatus, Scenedesmus quadricauda,* and *Pseudokirchneriella*
351 *subcapitata*) and microorganisms that colonized the artificial lake water. The communities were
352 exposed to 20 °C (control) and 28 °C (neat-lethal stress for Daphnia) temperatures for seven days
353 to mimic natural heatwave conditions and allow for eRNA accumulation in tank water.
354 Communities were reared in 8 L tanks containing artificial lake water [74] and were seeded with
355 a starting population of 50 parthenogenetic females of *Daphnia pulex* (clone from Illinois, USA;
356 40.24, -87.78). To generate dense *Daphnia* populations, tanks were kept in controlled chambers
357 for 45 days at optimal growing conditions: 20 °C, relative humidity of 50% and a photoperiod of
358 16:8 hour light:dark. *Daphnia* were fed 1.5 mL algae (1:1:1 ratio of *A. falcatus, S. quadricauda,*
359 and *P. subcapitata*) twice per week. After 45 days, the *Daphnia* populations in all tanks reached
360 a minimum of 500 individuals and were transferred to controlled experimental chambers pre-set
361 to the respective temperatures (20 °C and 28 °C).
362
363 *Sample collection*
364 Each temperature treatment had four tanks (biological replicates), and we collected four
365 eRNA and oRNA technical replicates per tank. We stirred the tanks for 60 seconds and filtered
366 500 mL of water through a 60 µm mesh to remove all *Daphnia* and ephippia. To capture eRNA,
367 the *Daphnia* free water was then filtered through a 0.7 µm glass microfiber filter. Each filter was
368 cut in half and placed in individual 1.5 mL microcentrifuge tubes containing 370 µL RLT buffer
369 (Qiagen) and 3.7 µL β-mercaptoethanol. Tubes were immediately stored at -80 °C until eRNA
370 extraction. Negative eRNA filtration control samples (500 mL of distilled water) were collected
371 at the beginning, middle and end of the sampling day, using the same methods. For each oRNA

372 sample, six live organisms (3 adults and 3 juveniles) per tank were haphazardly collected using a
373 sterile pipette and transferred to a 1.5 mL microcentrifuge tube containing 370 µL RLT buffer
374 (Qiagen) and 3.7 µL β-mercaptoethanol. To maintain the *Daphnia* heat stress transcriptional
375 signal, tubes were flash frozen in liquid nitrogen for 2 seconds, and immediately stored at -80 °C
376 until RNA extraction. The following system was used for sample identification:
377 RNAtype_Temperature&TankID (e.g. sample "eRNA_20T1" refers to eRNA collected at 20 °C
378 from tank 1).
379
380 *Extractions of eRNA and oRNA samples*
381 Samples of eRNA and oRNA were extracted using the RNeasy Mini Kit (Qiagen),
382 following the manufacturer's protocol with the following modifications. eRNA samples were
383 thawed on ice, gently vortexed for 5 seconds and then centrifuged for 3 minutes at 13,300 rpm to
384 separate the liquid from filter. To increase the eRNA yield, liquid from two technical replicates
385 was transferred into a 5 mL microcentrifuge tube containing 800 µL 70% EtOh and pipette
386 mixed. This mixture was transferred to an RNeasy spin column (700 µL at a time) and
387 centrifuged at 10,000 rpm for 15 seconds. We repeated this four times, until all remaining liquid
388 from the 5 mL microcentrifuge tube was transferred to the spin column. For oRNA, *Daphnia*
389 samples were thawed on ice and homogenized via sterile pestle and mortar mixer for 60 seconds.
390 We then added 400 µL of 70% EtOh to the homogenized tissue. The resulting mixture was
391 pipette mixed and transferred to an RNeasy spin column, where it was centrifuged at 10,000 rpm
392 for 15 seconds. The remainder of the eRNA and oRNA extraction procedures followed the
393 manufacturer's instructions, with RNA being eluted in 40 µL of DNase and RNase free
394 molecular grade water.

395

396 *DNA digestion*

397 Immediately following RNA extraction, an 18 µL aliquot of both RNA types underwent
398 two rounds of DNA digestion using the DNA-*free*™ DNA Removal Kit (Invitrogen) which
399 provided rDNase. We completed the first round of DNA digestion following the manufacturer's
400 instructions, but skipped the DNase inaction step. To ensure no DNA carryover, we completed a
401 second round of DNA digestion as follows: 2.7 µL of DNase I Buffer and 1 µL rDNase were
402 added and pipette mixed into each sample and the plate was gently vortexed, centrifuged, and
403 then incubated at 37 °C for 20 minutes. Following incubation, 2 µL of DNase Inactivation
404 Reagent was added to each well. The plate was incubated for 2 minutes at room temperature, and
405 centrifuged at 3700 rpm for 5 minutes to pellet the DNase Inactivation Reagent. The supernatant
406 containing RNA was transferred to individual sterile microcentrifuge tubes and stored at -80 °C
407 until library preparation.

408

409 *RNA-seq library preparation and sequencing*

410 We prepared libraries for whole transcriptome shotgun sequencing (RNA-seq) using the
411 Illumina Stranded Total RNA Prep with Ribo-Zero Plus kit and IDT® for Illumina® RNA UD
412 Indexes Set A, following the manufacturer's instructions. Equal volumes of RNA (post 2 rounds
413 of DNA digestion) from each technical replicate were pooled and used as input material (250 ng
414 and 26.4-65.56 ng for oRNA and eRNA, respectively) to prepare one biological replicate library
415 per tank. During library amplification, 12 and 17 PCR cycles were conducted for oRNA and
416 eRNA, respectively, to account for differences in starting material. Library quantification,

417 quality control and equimolar pool sequencing of the 16 libraries was conducted at the McGill
418 Genome Centre on one Illumina NovaSeq 6000 S4 lane using paired-end 100 bp reads.
419
420 *Contamination prevention*
421 We submerged all experimental and sampling equipment in a 20% bleach solution for 15
422 minutes and rinsed thoroughly five times with distilled water prior to use. All eRNA samples
423 were processed in a pre-PCR clean laboratory dedicated to low quality environmental and
424 ancient DNA/RNA samples. Prior to entering the pre-PCR sterile laboratory, researchers entered
425 a decontamination room to change into the dedicated clean lab coats and put on face masks,
426 hairnets and lab clogs with shoe covers. The laboratory workbench was soaked in a 20% bleach
427 solution for 10 minutes and RNase WiPER™ (iNtRON Biotechnology) and 20% bleach was
428 used to thoroughly wipe pipettes, vortex mixers and centrifuges. Aerosol filter barrier pipette tips
429 were used to prevent cross-contamination of samples. Filtration negative control samples and
430 molecular negative control samples (consisting of reagents or DNA/RNA free molecular grade
431 water) were processed at each step alongside eRNA samples, and were free of contaminating
432 nucleic acids as identified by failed PCR amplification using universal COI primers known to
433 amplify *Daphnia* DNA [75]. Successful DNA digestion of each eRNA and oRNA sample was
434 verified by failed PCR amplification of RNA post-DNA digestion x2 using universal COI
435 primers known to amplify *Daphnia* DNA [75]. RNA-seq libraries were prepared from filtration
436 and molecular negative control samples and were free of contamination as indicated by quality
437 control checks and failed amplification conducted at the McGill Genome Centre.
438
439 *Bioinformatic pipeline and statistical approaches*

440 Raw FASTQ files underwent initial sequencing quality inspection using FastQC [76].
441 Low quality sequences and adapters were removed using Trimmomatic [77] (ILLUMINACLIP:
442 lluminaStrandedTotalRNA_adapter.fa:2:30:15 TRAILING:30 HEADCROP:1 MINLEN:90). All
443 statistical analyses were conducted using R (R Core Team, 2021, R version 4.1.2). With default
444 parameters, STAR [78] was used to map all eRNA and oRNA reads that passed quality control
445 and trimming to the *Daphnia pulex* reference genome [45]. Using Kallisto [79], we also mapped
446 eRNA reads to the reference genomes and transcriptomes of species known a priori to persist in
447 the communities and highly abundant species identified by alignment of eRNA reads to NCBI nr
448 database [80–87]. FeatureCounts [88] was used on the STAR resulting BAM file to quantify
449 gene counts. Only those genes with at least one count in three out of four eRNA 20 or 28 °C
450 samples were kept and considered in the subsequent differential expression (DE) analysis.
451 DE analysis was conducted with DESeq2 [89], comparing all 20 °C samples versus all 28
452 °C samples, for both eRNA and oRNA samples. A gene was determined to be significantly
453 differentially expressed if it had a false discovery rate (FDR) adjusted p-value < 0.1. WEGO 2.0
454 [90] was used to classify GO terms (acquired from [45]) to all annotated *D. pulex* DEGs. Gene
455 ontology enrichment analysis was conducted using topGO [91] by comparing GO terms of the
456 relevant genes to the genomic background. A FDR corrected p-value < 0.05 indicated significant
457 enrichment. A principal component analysis (PCA) of the regularized log (rlog)-normalized
458 eRNA and oRNA gene expression profiles (300 most variant genes) at both optimal and heat
459 stressed conditions was conducted using DESeq2's plotPCA function [89]. Heatmaps showing
460 the relative expression (Z-score calculated for each gene) of all significantly differentially
461 expressed genes between 20 and 28 °C, in both *D. pulex* oRNA and eRNA, and all other species
462 in eRNA, were generated using pheatmap [92].

463 We used the SqueezeMeta metatranscriptomics pipeline for the eRNA community-wide
464 analysis [46]. Briefly, metatranscriptomes were assembled in sequential mode and contigs were
465 assembled using Megahit [93]. Diamond was used to align all contigs to the GenBank nr and
466 KEGG databases for taxonomic assignment and KEGG ID annotation, respectively [94,95]. The
467 SQMtools R package was used to further analyze the SqueezeMeta data [96]. Stacked-bar plots
468 were created to represent the most relatively abundant taxa. We conducted non-metric
469 multidimensional scaling analysis of all KEGG IDs to visualize the community functional
470 profiles.
471
472 **Data Availability**
473 Sequences for all RNA-seq libraries have been uploaded to the NCBI SRA database and will be
474 made public upon acceptance of this manuscript.
475
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803 **Figures and Tables**

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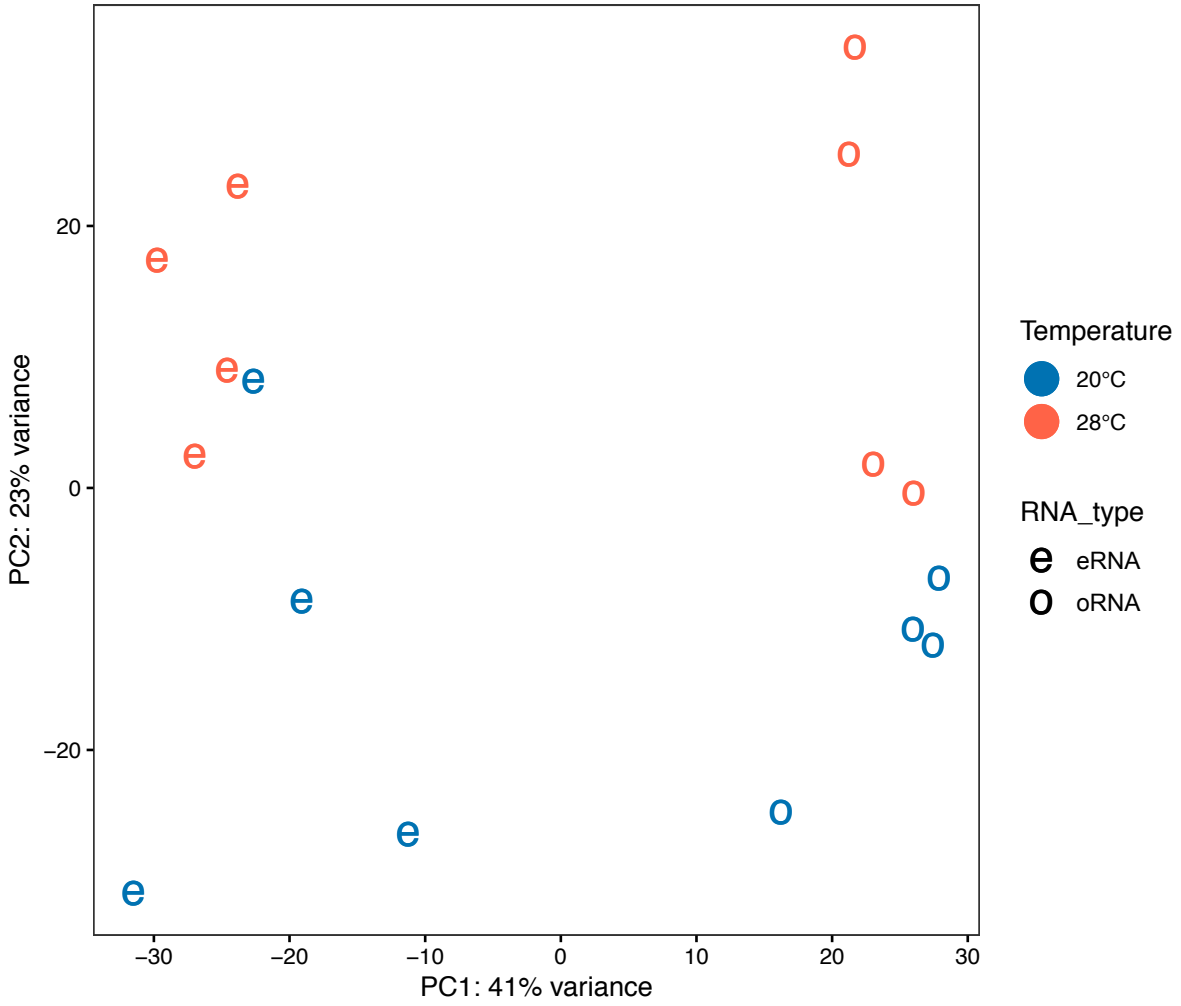

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806 **Fig. 1.** Principal component analysis (PCA) of the top 300 *Daphnia pulex* genes, as ranked by
807 variance. Prior to the PCA, genes were filtered to include only those genes that had ≥ 1 count in
808 either 3/4 20 °C or 28 °C samples. Sample eRNA_20T1 clusters more closely with 28 °C
809 samples than 20 °C samples. PCA revealed separation by RNA type and temperature across the
810 PC1 and PC2, respectively. eRNA refers to extra-organismal RNA released by *D. pulex* into the
811 environment, whereas oRNA refers to organismal RNA extracted directly from *D. pulex* tissue.
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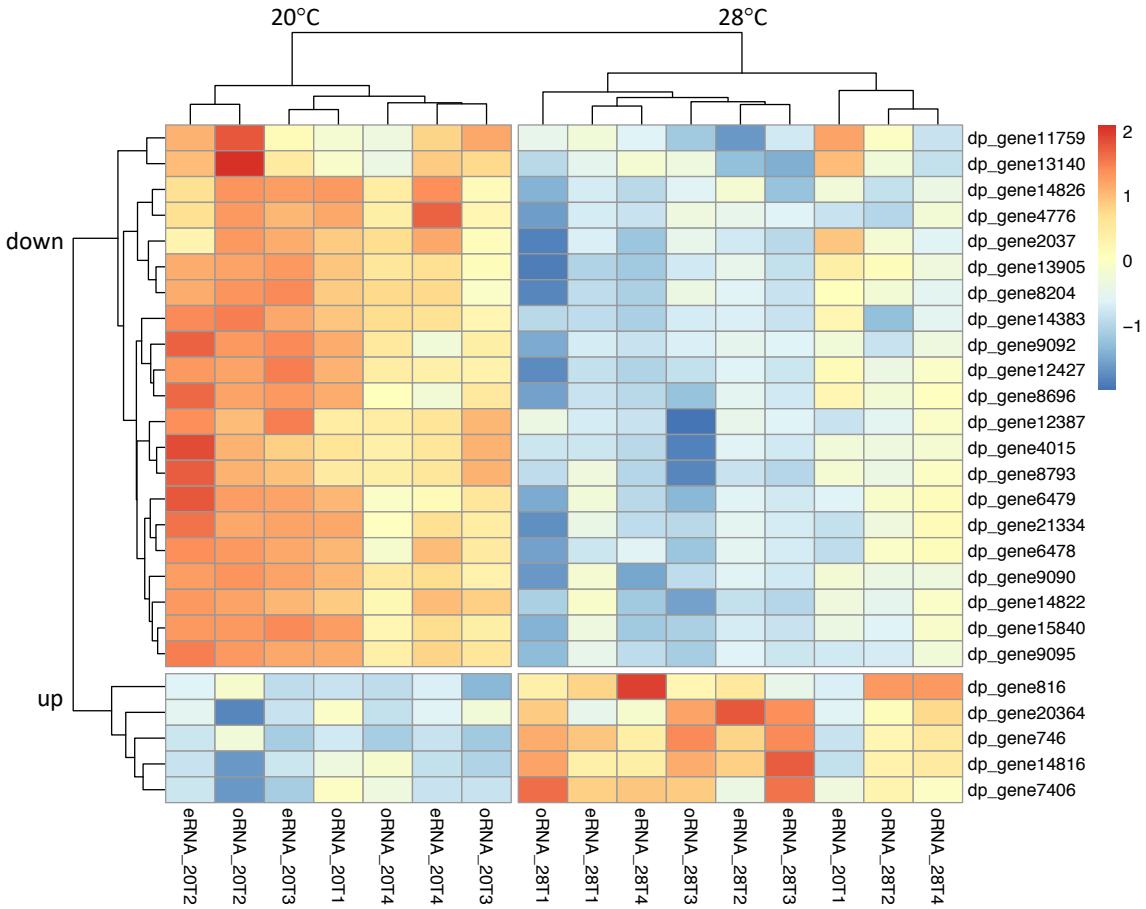

Fig. 2. Heatmap showing the relative expression (Z-score calculated for each gene) of all commonly significantly differentially expressed (DE) *Daphnia pulex* genes (false discovery rate adjusted p-value <0.1) between 20 °C and 28 °C, in both environmental RNA (eRNA) and organismal RNA (oRNA). DE analysis was conducted only for those genes that had ≥ 1 count in either 3/4 20 °C or 3/4 28 °C samples. Hierarchical clustering analysis revealed four groups: 20 °C and 28 °C samples, and up and down regulated genes. Sample eRNA_20T1 clusters with 28 °C samples. Colors indicate levels of relative expression, with blue and red indicating low and high, respectively. DE statistics and functional description of the genes are provided in Table S1.

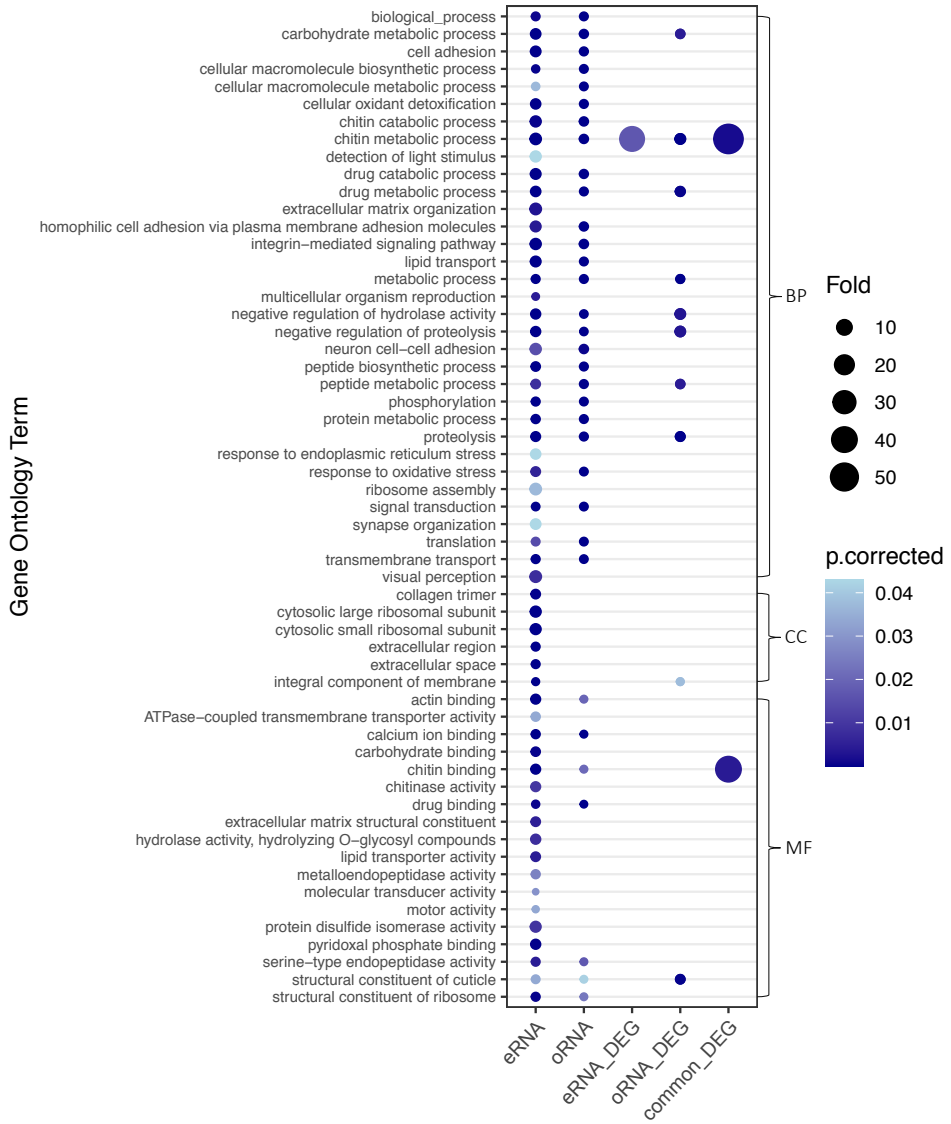

Fig. 3. Bubble plot of the Gene Ontology (GO) enriched terms associated with *Daphnia pulex* eRNA and oRNA detected genes, and eRNA, oRNA and commonly identified differentially expressed genes (DEG). The eRNA genes originate from extra-organismal *Daphnia* RNA. We used a false discovery rate (FDR) corrected p-value <0.05 in topGO to identify GO terms as significantly enriched compared to the genomic background. The bubble color indicates the FDR corrected p-value for the weight test statistic, while the size is proportional to the fold change between expected and observed genes annotated with GO term. The biological processes, cellular components and molecular functions GO domains are represented by BP, CC, and MF, respectively. Only those GO terms enriched for eRNA genes are shown. Full list of GO terms enriched for oRNA genes is provided in Fig. S3.

Table 1. Problems associated with using environmental transcriptomics based on eRNA to monitor the health of ecological communities and potential experimental (E), molecular (M), sequencing (S) and bioinformatic (B) solutions.

Problems	Description of problems	Possible solutions	References
Poor understanding of the ecology of eRNA	Little is known about the origin, state, persistence and degradation of eRNA in the environment.	E: Perform experiments that address fundamental questions of the ecology of eRNA (i.e. What are major sources of eRNA? What state is eRNA in? What factors influence eRNA persistence? How do abiotic factors influence eRNA decay rates?)	[11]
No understanding of environmental transcriptomics in natural ecosystems	No information available about how our results using environmental transcriptomics will translate to natural ecosystems	E: Perform mesocosm experiments using more complex, multi-trophic communities E: Perform studies using ecosystem wide manipulations E: Perform studies in natural ecosystems	
Rapid degradation of eRNA	eRNA is thought to degrade too rapidly to extract and quantify, but studies indicate that eRNA is detectable for up to 57 hours post organism release	E: Filter water as soon as possible (ideally < 4 hours) E: Freeze samples at -80 °C directly after filtration M: Keep samples on ice during extraction M: Minimize free-thaw cycles	[17,19,21]
Low concentration of eRNA	eRNA samples have much lower RNA concentrations than samples collected from an organisms tissue (i.e. 1 ng/µL vs 200 ng/µL)	E: Increase RNA yield by filtering large volumes of water (> 500 mL); EM: Collect additional technical replicates and pool them during the extraction step; M: Use RNA-seq library prep kits designed for low input starting material	
Low proportion of mRNA	mRNAs are of interest for gene expression analyses as they encode for proteins, but they account for < 5% of total RNA. Non-coding ribosomal and small RNAs account for > 85%	M: Perform rRNA depletion to remove rRNA. However, many commercial kits are optimized to remove human, mouse, rat and bacteria rRNA, and may not deplete rRNA from all species; M: Perform poly(A) enrichment to retain only eukaryotic mRNA. This will eliminate prokaryotic RNA, ribosomal and small RNAs, which make up the majority of total RNA B: If rRNA persists, use it to perform taxonomic identification analysis	[97–100]
Low eRNA recovery	eRNA samples are composed of extra-organismal RNA from macro-organisms and whole microorganism. Thus, eRNA reads will be in the minority (estimated <1%), resulting in low power of bioinformatic analyses to detect eRNA based changes in gene expression	E: Pre-filter water with fine mesh to size-selectively remove organisms; E: Use large filter pore size to reduce capture of microorganisms (i.e. > 0.2 µM); E: Sample eRNA from ecosystems that are densely populated with target species M: Perform rRNA depletion on total RNA samples; M: Perform poly(A) enrichment to eliminate prokaryotic RNA; S: Perform deep sequencing to increase eRNA reads (i.e. > 150 million reads per sample)	
Low eRNA read depth	eRNA samples are extremely diverse, resulting in reads from taxa across the trophic chain. Transcript abundance in a sample will influence read depth, and if many micro-organisms are present, eukaryotic genes will have low read depth. Current molecular methods for enrichment and depletion are optimized for organismal, not diverse environmental samples.	M: Create low input poly(A) enrichment methods. Current enrichment methods are designed for organismal RNA from tissue samples and require high concentration of input RNA; M: Increase eukaryotic gene read depth through poly(A) enrichment; MB: Create rRNA depletion methods that remove rRNA from all taxa as current depletion methods are optimized to remove single taxa and bacterial rRNA. If unable to remove all rRNA from eRNA samples, researchers could use rRNA reads for taxonomic information; M: Perform rRNA depletion; M: Amplify biomarker genes via targeted (qPCR, ddPCR) approaches to analyze specific biomarker genes	[13]
Difficulty characterizing read function due to lack of eukaryotic reference genomes	Reference genomes are used to functionally and taxonomically characterize reads. eRNA samples will capture eRNA from eukaryotes across the trophic chain, but genomes are available for only 0.4% of eukaryotic species.	ES: Sequence all eukaryotic species (e.g. Earth BioGenome Project) B: *De novo* assembly of reads into longer scaffolds, which enables the identification of homologs.	[101,102]

Lack of baseline in natural ecosystems to compare against	Differential gene expression analyses are conducted by comparing treatment samples (i.e. stressed community) against a control 'baseline' (i.e. health community). In natural ecosystems it is difficult or often not possible to obtain a baseline control as many stressors act simultaneously on a system	E: Conduct time series eRNA sampling of the same environment and compare time points; E: Compare environments with similar abiotic conditions. If healthy, one could serve as a baseline to compare against; EM: Couple eRNA with targeted PCR approaches (qRT-PCR, ddPCR) and biomarker genes to infer physiological states of specific species; EB: Reconstruct mock communities under neutral conditions as a baseline; B: The cellular stress response is triggered by general stress and as such genes and pathways involved could be broadly indicative of community stress; B: Use housekeeping genes with consistent expression levels as baselines to compare against; B: Supervised machine learning models could be trained with existing metatranscriptomic, transcriptomic and metagenomic data that have identified functional terms, metabolic pathways and taxa associated with stressors. Environmental transcriptomic data could be used as input and the model would predict the stressors involved and health of the community	[13,71–73,103]
False detection of differential transcript expression due to changes in population densities	Between environments, species across the trophic chain exhibit differences in abundances. RNA transcript abundance varies with DNA abundance. Thus, changes in RNA transcript abundance, independent of differential expression, could be recovered.	EB: Collect and sequence eDNA in parallel. eDNA can acts as a species abundance estimator, independent of transcript abundance, that we can normalize eRNA reads to. Allows us to track whether the proportion of eRNA reads follows species abundance, or is up or down regulated; B: 'Taxon-specific scaling' accounts for variation in abundances by separating and then normalizing metatranscriptomic data into organism-specific bins. A differential expression analysis could then be performed after the normalized matrices are recombined into community wide data	[13,22,70,72]