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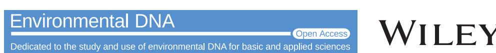
COMMENTARY



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Messenger RNA typing of environmental RNA (eRNA): A case study on zebrafish tank water with perspectives for the future development of eRNA analysis on aquatic vertebrates

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Abstract

As an alternative/supplement to conventional biodiversity survey methods, environmental DNA (eDNA) analysis has developed rapidly during the past decade, and is widely used for the biomonitoring of wildlife. However, the interpretation of eDNA results has been limited to the presence/absence or biomass/abundance of the target species because of uncertainty regarding the dynamics of eDNA in natural environments. This limitation may be ameliorated by targeting environmental RNA (eRNA). RNA is more prone to degrade than DNA and the pattern of messenger RNA (mRNA) expression changes depending on physiological conditions, meaning that the presence or concentration of mRNA could reflect the organism's presence with higher temporal resolution and provide information beyond simple presence/absence. Technical developments in the detection of eRNA focusing on mRNA with these distinct features could permit the advanced usage of genetic materials in water. In advancing this technique, we initiated this study asking that if we can detect elevated levels of eRNA whose genes are specific to a tissue source (e.g., gills or skin), then could not we infer the tissue origin of the genetic material detected. To this end, we developed gene-specific primer sets for the target genes with biased expression in the gills, skin, and intestine, and conducted reverse transcription–polymerase chain reaction on zebrafish breeding tank water samples, obtaining positive results for all assays. The result of our experiment confirmed that the specific target tissues can be the source of genetic materials detected in water and with that we offer a proof of concept for eRNA analysis targeting specific mRNAs of aquatic vertebrates. In this commentary, we provide information on the experimental steps used for mRNA typing of eRNA from zebrafish as well as the limitations and challenges of this technique and the prospect of mRNA typing of eRNA in the future.

KEYWORDS

biological monitoring, conservation of natural resources, gene expression, polymerase chain reaction, reverse transcription, RNA

Kenji Tsuri and Shizuya Ikeda contributed equally.

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1 | INTRODUCTION

Biological monitoring in the natural environment relies heavily on the use of survey methods, some of which may potentially be invasive through the capturing and handling of animals, and many survey methods also require taxonomic expertise in species identification (Cilleros et al., 2019; Gunzburger, 2007; Hill et al., 2005; Pidgeon, 2004; Shaw et al., 2016). As an alternative/supplemental survey method, environmental DNA (eDNA) analysis has developed rapidly during the past decade, and it is widely used for the biomonitoring of aquatic macroorganisms (Barnes & Turner, 2016; Goldberg et al., 2016; Thomsen, Kielgast, Iversen, Møller, et al., 2012; Tsuji et al., 2019). This method detects species by analyzing DNA released by organisms into the environment, and it does not require morphological taxonomic expertise because it identifies species based on their DNA sequences. eDNA analysis has several advantages over traditional survey methods such as a lower cost (Evans et al., 2017; Sigsgaard et al., 2015) and noninvasiveness, and it has high efficiency in detecting nocturnal and low-density species, including rare and invasive species (Fukumoto, Ushimaru, & Minamoto, 2015; Harper et al., 2019; Seymour, 2019; Yamamoto et al., 2016).

Although eDNA analysis has been widely used in many studies, the analysis results always include uncertainties. For example, when and where the detected eDNA was released are unknown (Barnes et al., 2014; Deiner & Altermatt, 2014; Dejean et al., 2011; Jane et al., 2015). Moreover, it cannot be judged whether the eDNA was released from living or dead individuals (Darling & Mahon, 2011; Merkes et al., 2014). Turner et al. (2015) reviewed studies of eDNA and found a maximum persistence time of 25 days (Barnes et al., 2014; Dejean et al., 2011; Goldberg et al., 2013; Pilliod et al., 2014; Thomsen, Kielgast, Iversen, Møller, et al., 2012; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012). Merkes et al. (2014) reported that eDNA released by silver carp caracasses remains detectable for at least 28 days. Therefore, eDNA may have been transported over long distances or released from dead individuals, which could lead to false-positive detection. Moreover, typical eDNA analyses do not provide information related to physiological state (but see Bylemans et al., 2017 for the estimation of reproduction) of the species.

Meanwhile, the pattern of messenger RNA (mRNA) expression changes depending on physiological conditions, such as sexual maturity (Chen & Ge, 2012) and reproduction (Shahjahan et al., 2010; Zhang et al., 2015; Zohar & Mylonas, 2001), and those that result from environmental stress (e.g., hypoxia: Rabergh et al., 2000; Rytkönen et al., 2014). Given this variable expression of mRNA, it could be possible to infer useful information for ecological or conservation purposes from environmental samples other than the presence/absence of organisms if eRNA analysis is applicable to aquatic vertebrates to extrapolate the physiological state of organisms noninvasively. By focusing on key genes such as reproduction-relating ones, it would be possible to develop eRNA markers to infer the progress of gonad maturation and spawning. Because some genes are expressed at elevated levels in a subset of tissues (i.e., tissue-specific genes) relative to the baseline expression across all tissues (Yang et al., 2018; Yu et al., 2006), it would also be possible to clarify the dynamics of eDNA by determining its source, which is one of the most basic but unanswered question in this field (Barnes & Turner, 2016). The difference in the features in the external body surface among fish species is likely to affect the shedding rate of eDNA (Sassoubre et al., 2016). Clarification of the source tissues of eDNA for each species could be recognized as fundamental for further elucidation of the species difference in their detection sensitivity.

This study was conceptualized to provide a proof of concept of the extension of eRNA analysis to vertebrate species to retrieve biological information other than simple presence/absence data from environmental samples such as water. We aimed to use eRNA analysis to confirm the types of tissues that were potential sources of fish eDNA in water by targeting genes with biased expression in the gills, skin, and intestine. Some reviews suggested that the sources of eDNA could be skin cells, mucus, scales, urine, feces, saliva, and gametes (Deiner et al., 2017; Handley, 2015; Harper et al., 2019; Rees et al., 2014; Thomsen & Willerslev, 2015; Turner et al., 2015). The detection of mRNAs that are highly expressed in specific tissues from eRNA samples could provide evidence of the source of eDNA/eRNA as we applied filtration method to retrieve eRNA from the water with 0.45 μm pore-sized filter (see Section 2 for details) which mainly captures the genetic materials as cell form. Specifically, we developed specific primer sets for genes with biased expression in the skin/epidermis, gills, and intestines and conducted Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) using water samples from a zebrafish (*Danio rerio*) breeding tank. We obtained positive results in all assays as expected, and the success of our experiment could represent evidence of the practicality of gene-specific eRNA analysis in future studies. For instance, if eRNA could be used to detect genes expressed solely in gametes or reproductive tissues during spawning, similar to the detection of other tissue-specific genes in our trial, very useful applications could result, such as identification of spawning habitat or detection of breeding invasive species populations.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All animal experimental procedures adopted in this study complied with the relevant laws and guidelines in Japan. Based on the current rules, approval of animal experiments using fish was not required, but we followed the instructions suggested by Japanese animal welfare regulations and the "Act on Welfare and Management of Animals" (Ministry of Environment of Japan) for husbandry and handling of fish.

2.2 | Target species and genes

Zebrafish was used as a model in this study because its gene expression has been extensively studied, making it preferable for a trial study of eRNA analysis in vertebrate species. Several genes

with biased expression in specific tissues were selected as target genes. *Chloride channel 2c* (*clcn2c*) is a Cl^- channel that belongs to the chloride channel protein 2 family of chloride channel/transport proteins, and its expression is relatively abundant in the gills (Pérez-Rius et al., 2015). *Fatty acid binding protein 2* (*fabp2*) is believed to be involved in the intracellular transport of fatty acids that are insoluble in water, and its expression is especially abundant in the intestine (Baier et al., 1996; Sharma et al., 2004). *Mucin 5.2* (*muc5.2*) is involved in the production of mucin, which is the primary component of body surface mucus. Expression of this gene is especially abundant in the skin and pharynx/esophagus (Jevtov et al., 2014). *Plectin a* (*pleca*) is involved in the production of plectin, a component of structures in which epithelial cells adhere to the basement membrane. This gene is mainly expressed in the epidermis (Li et al., 2011). *Keratin 5* (*krt5*) is involved in the production of keratin, which constitutes the cytoskeleton of the epithelium. This gene is also mainly expressed in the epidermis (Li et al., 2011). *Beta 2-microglobulin* (*b2 m*) encodes a protein found on the cell surface in a form noncovalently associated with the alpha chain of the class I major histocompatibility complex molecules (Ono et al., 1993). *b2 m* is one of the most commonly used housekeeping genes in cellular physiology (Casadei et al., 2011; McCurley & Callard, 2008). The housekeeping gene was included with the target genes as a positive control to confirm successful workflow in the experiments, as it should be always detected if zebrafish eRNA is present in the sample.

2.3 | Primer development

Species- and gene-specific primer sets for the target genes of zebrafish (Table 1) were designed. The fish tank used for this study contained no vertebrates other than zebrafish, but all primer sets were designed to be species-specific to the target species as much as possible. The mRNA sequences of the target genes of zebrafish and the other nontarget species were collected from the nucleotide sequence archive of the National Center for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/nucleotide/). All Cyprinidae species with target gene sequences registered at NCBI were set as nontarget species, including *Carassius auratus*, *Cyprinus carpio*, *Sinocyclocheilus anshuiensis*, *Sinocyclocheilus graham*, and *Sinocyclocheilus rhinocerous*. Primer pairs were designed to have at least two nucleotide substitutions within five bases from the 3′-end against the other nontarget species in at least one of the forward and reverse primers, and the opposite primer was set on the exon–exon junction of the target gene to suppress the undesirable amplification of genomic DNA (gDNA) which includes intron sequences. The primer sets were tested by in silico PCR using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the results supported the species and gene specificity of each primer set. While the currently available sequence database for our target gene regions is insufficient to establish definitive species specificity, conducting an in silico test is still the best method available at this time. The process of confirming the primers we developed were for tissue-derived RNA is described in Supporting Information.

2.4 | Detection of eRNA in tank water

Two 20-L experimental tanks were filled with 12 L of aged tap water. On the next day, 60 adult zebrafish (approximately 6 months after fertilization; no sex identification; wet weight, approximately 0.30 g; total length, approximately 30 mm) were transferred to one of the tanks, which was designated the "fish tank." The tank lacking fish was designated the "blank tank." Both tanks were aerated and maintained at 25°C throughout the experiment. A 12-hr/12-hr light/dark cycle was employed. Prior to the experiment, all equipments such as air stones, air tubing, and tanks were treated with 10% bleach solution for at least 5 min and thoroughly rinsed with running tap water. Water was collected from both tanks 7 days after the introduction of zebrafish. Specifically, 3.0 L of water from the fish tank was collected into 10-L plastic container by scooping water with a disposable polyethylene cup. The water sample was stirred well and dispensed in 1-L amounts into disposable polyamide packs to create triplicate subsamples. We also collected 1 L of water from the blank tank, giving four water samples in total. The water samples were immediately filtered using a Sterivex filter cartridge with a nominal pore size of 0.45 μm (SVHV010RS; Merck). After filtration, each cartridge was sealed with luer caps (VRMP6 and VRSP6, ISIS Co., Ltd.) on both the inlet and outlet ports. RNA extraction was performed immediately after filtration. It has been suggested that eDNA includes DNA in multiple states, such as extracellular and intracellular, and that eDNA collected on filters is suggested to be in the cell form (Turner et al., 2014; Wilcox et al., 2015). Considering RNA is prone to degrade outside the cell into shorter fragments, permitting it to pass through filter pores, extracellular mRNA is not expected to be recovered by the filtration method. Based on this, we assumed that both the eDNA and eRNA were collected in the intracellular state by the filtration method and this shared the same origin. The eRNA extraction and RT-PCR processes are shown in Supporting Information.

3 | RESULTS & DISCUSSION

In the in vitro test of the designed primers, all target genes were confirmed to be amplified from tissue-derived samples (Table 2; Figure 1). However, only *clcn2c* (gills) and *muc5.2* (skin) were confirmed to have clear tissue-specific expression. Conversely, *fabp2* (intestine), *pleca* (epidermis), and *krt5* (epidermis) were revealed to be expressed in unexpected tissues, that is, visible band on the gel. Specifically, *fabp2* was expressed in the intestine, brain, and liver. *pleca* was expressed in all tissues. *krt5* was expressed in the gills, liver, intestine, and skin.

The target genes were detected by RT-PCR using fish tank eRNA (Table 2; Figure 2). *b2 m*, *pleca*, and *krt5* were detected in all water sample replicates. *clcn2c*, *fabp2*, and *muc5.2* were detected in at least

TABLE 1 Target genes and primer sequences

Gene name	Gene Symbol	The most abundant tissue	Primer sequence	Product length
Chloride channel 2c	*clcn2c*	Gills (Pérez-Rius et al., 2015)	F: 5′-CACTGAACGAGTTGTGTAAGGCC-3′ R: 5′-GCAGTTTGTGCAGCTCACACA-3′	135 bp
Fatty acid binding protein 2	*fabp2*	Intestine (Sharma et al., 2004)	F: 5′-CTCACAGGATCCTGGGTCATAGA-3′ R: 5′-AATGGTGAACTTGTACAGAGCTATAGC-3′	123 bp
Mucin 5.2	*muc5.2*	Skin, pharynx/esophagus (Jevtov et al., 2014)	F: 5′-CTACATCCTCACTAAGCACTCTAACG-3′ R: 5′-CAGTGTCACAACACCATCAGAAGTA-3′	157 bp
plectin a	*pleca*	Epidermal (Li, Frank, Thisse, Thisse, & Uitto, 2011)	F: 5′-GTCAGCTCTGGTTTGGATGGG-3′ R: 5′-GAAATTCCAGGAGTTGATAAGCGTG-3′	132 bp
keratin5	*krt5*	Epidermal (Li et al., 2011)	F: 5′-CGAGACAAAATGGAGTTTATTGCAAGAA-3′ R: 5′-TATTTGTTCTTGAAGTCCTCCACCAA-3′	180 bp
Beta-2-microglobulin	*b2m*	Whole organism (McCurley & Callard, 2008)	F: 5′-GGGAAAGTCTCCACTCCGAAAG-3′ R: 5′-GGTCTGCTTGGTGTCCGAC-3′	159 bp

TABLE 2 Primer test and RNA detection from fish tank water

Gene name	Gene Symbol	The most abundant tissue	Detection of the most expressed tissue	Tissue specificity of genes	Detection from fish tank
Chloride channel 2c	*clcn2c*	Gills (Pérez-Rius et al., 2015)	☒	☒	☒☒☐
Fatty acid binding protein 2	*fabp2*	Intestine (Sharma et al., 2004)	☒	☐	☒☒☐
Mucin 5.2	*muc5.2*	Skin, pharynx/esophagus (Jevtov et al., 2014)	☒	☒	☒☒☐
Plectin a	*pleca*	Epidermal (Li et al., 2011)	☒	☐	☒☒☒
keratin5	*krt5*	Epidermal (Li et al., 2011)	☒	☐	☒☒☒
Beta-2-microglobulin	*b2m*	Whole organism (McCurley & Callard, 2008)	All tissues	N.A.	☒☒☒

Note: The successful detection of each gene in its "most abundant tissue" reported in previous studies is shown in the fourth column. If the pattern of expression of a gene among tested tissue types exhibited a clear bias to the "most abundant tissue," then the gene was confirmed to be tissue-specific, as indicated in the fifth column. The positive detection of each gene in triplicated water samples from fish tank water is shown in the sixth column.

two replicates. The sequences of the PCR products were verified using Sanger sequencing.

The water sample from the blank tank were negative for all genes. The No Reverse Transcriptase control (No-RT control) was negative in all PCRs, and it was determined that gDNA did not contaminate any complementary DNA (cDNA) samples. Additionally, all No Template Controls (NTCs) tested negative.

The detection of two genes with biased expressions in gills (*clcn2c*) and skin (*muc5.2*) suggests these tissues are part of the source of eDNA/eRNA. Concerning *fabp2*, the gene was confirmed to be expressed in tissues other than the intestine in vitro, and Sharma et al. (2004) similarly observed low-level expression of the gene in other tissues, although its expression was highest in the intestine. Therefore, *fabp2* does not have perfect specificity for the intestine, but the tissue is likely to be another source of eDNA/eRNA. Contrarily, *pleca* and *krt5* did not exhibit tissue-specific expression, and thus, they are unlikely to be effective target genes for determining the tissue sources of eDNA/eRNA. We detected target mRNAs in eRNA samples, but there was variation among the triplicate water samples from the same tank. There might be random sampling error, implying some of the focal mRNAs were very rare even in the tank water. This is not the case for eDNA analysis targeting the mitochondrial barcoding region, which is typically abundant especially in the tank experiments. This suggests the need for an increase in the number of replicates and in the volume of water samples relative to eDNA analysis. Further development of better sampling protocols with higher recovery rates is also desirable. It seems highly unlikely that detection of any mRNA would be 100% under typical conditions even in a case of tank experiment. Though there were some difficulties in the detection, to the best of our knowledge, this is the first

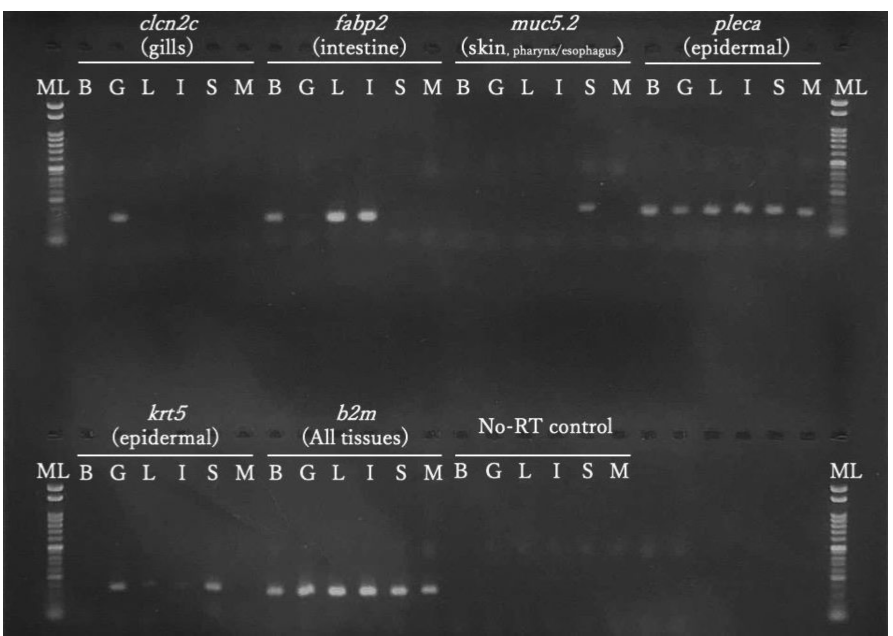

FIGURE 1 Gel electrophoresis image of each target gene in each tissue (B = brain, G = gills, L = liver, I = intestine, S = skin, *M* = muscle, ML = marker ladder)

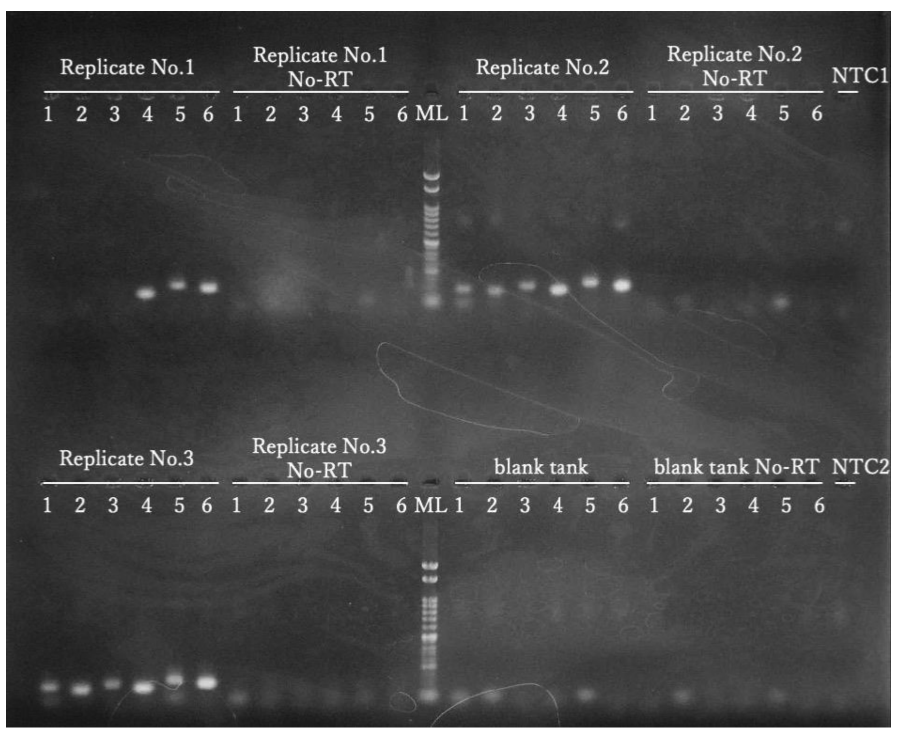

FIGURE 2 Gel electrophoresis image of environmental RNAs from tank water samples (1 = *clcn2c*, 2 = *fabp2*, 3 = *muc5.2*, 4 = *pleca*, 5 = *krt5*, 6 = *b2 m*, No-RT = No-RT control, NTC = No template control)

study to successfully retrieve information other than the presence/absence of aquatic vertebrate species via eRNA analysis using water samples, that is, a proxy for the source tissue of eDNA/eRNA, by focusing on several target mRNAs. This result is a proof of concept of the practicability of eRNA analysis targeting on mRNA which has potential to assess the physiological status of aquatic vertebrates, and further development of this method might contribute to a more detailed evaluation of habitat suitability by focusing on key genes which reflect environmental condition.

4 | PERSPECTIVES

The present result identified the gills, skin, and intestine as sources of eDNA, and these data could represent further confirmation of the authenticity of the sources estimated in previous research. Further detection of tissue-specific genes in water samples using the developed method will help to further elucidate the sources of eDNA/eRNA. Moreover, the present success in mRNA typing of eRNA could be a milestone as a starting point of the development of new eRNA

analyses for inferring physiological status of aquatic vertebrates. For instance, genes relating to reproduction (e.g., *KISS receptor a* and *Kisspeptin* 2 identified in *Takifugu niphobles* in Shahjahan et al., 2010, *follicle stimulating hormone subunit beta* and *luteinizing hormone subunit beta* identified in *D. rerio* in Zhang et al., 2015) would be the good target markers to infer the occurrence of reproduction activity. In a previous study, the ratio of nuclear DNA and mitochondrial DNA in water was used as a marker for reproduction based on the knowledge that the sperms have the higher ratio than the other somatic cells (Bylemans et al., 2017). The usage of eRNA analysis targeting on specific mRNAs might provide more sensitive assay to infer the progress of reproduction by focusing on some related genes on the expression pathway for reproduction. Moreover, RNA is more prone to degrade than DNA, the feature also might contribute to increase the sensitivity temporally and spatially in conjunction with quantitative RT-PCR method. As a practical example of the application of eRNA analysis in the management of invasive species, this technique could be used to detect the "establishment" of a new population of invasive species, that is, not only detecting the introduction of individuals but also the signature of reproduction. The establishment of invasive alien species is defined as the emergence of an alien species in a new habitat, the successful production of viable offspring, and the likelihood of continued survival (CDB Secretariat, 2002). Therefore, noninvasive monitoring could be performed to produce sufficient information to judge establishment success based on the definition regarding a target invasive species using eRNA and eDNA analyses.

The overwhelming shortage of mRNA sequence and transcriptome data of macroorganisms is a major issue for future eRNA analyses. In this study, the species and gene specificities of the newly designed primer set were confirmed using Primer-BLAST. However, without sufficient sequence information on the related organisms with target genes registered in the database, we cannot conduct practical assessments of the specificities because of the possibility of unintended amplification of mRNAs from other unregistered species and genes in the case of field use. Expansion of the mRNA sequence database is essential for the application of eRNA analysis for a variety of genes in multiple species in the field. Further investment on enriching (or improving) the database would allow greater freedom of choice of target gene of interest for researchers/managers as they can select event-related or physiological condition-dependent mRNAs. Meanwhile, there is a need for compiling a list of genes that are retrievable from water samples using RNA-seq so we can correctly identify candidate genes compatible with eRNA analysis. The potential use of eRNA analysis as more than a simple presence/absence of species will revolutionize ecological monitoring methods by making it easier to obtain useful data for biological conservation.

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CONFLICT OF INTEREST

There are no conflicts of interest to declare.

AUTHOR CONTRIBUTION

K.T., S.I., and H.Y. conceived and designed the research. Y.S. and T. M. significantly contributed to the refinement of the original research plan. K.T., S.I., and T. H. led the experiments. K.T. and H.Y. wrote the first draft of the manuscript, and all authors discussed the results and contributed to the development of the manuscript.

DATA AVAILABILITY STATEMENT

All data obtained in this study are disclosed in the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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