Title

Environmental RNA mitigates fish ghosts related to fish feeds for aquaculture in molecular ecological survey in a bay.

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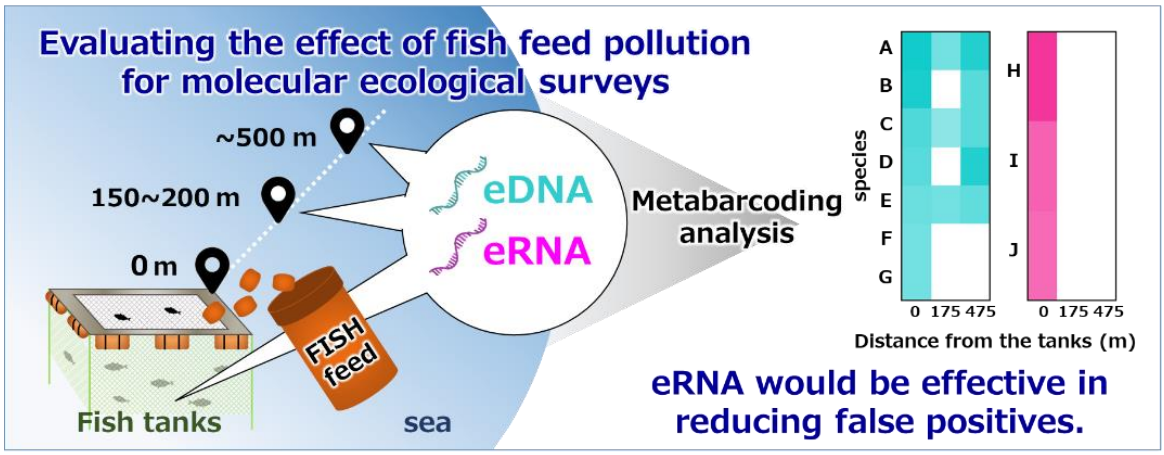
Abstract

The use of environmental DNA for molecular ecological surveys has become widely utilized in ecological assessments of various water environments, from rivers to coastal and marine areas. However, it is important to consider the scenarios of potential contamination of environmental nucleic acids in interpreting the results. In this study, we analyzed the fish species present in the feed that may affect surveys near aquaculture facilities and fish processing plants using metabarcoding analysis of environmental DNA/RNA, and simultaneously evaluated whether the fish species present in the feed used for aquaculture in a heavily cultivated bay could be detected through metabarcoding analysis, thus evaluating whether fish species present in the feed could be detected in ecological surveys in the marine environment. A total 51 fish species (DNA: 46, RNA: 31) were detected in three type of fish feeds; detected species in DNA were more than those in RNA and common species that detected in both of feeds and water samples in the bay is remarkably more in eDNA than eRNA. Moreover, the number of those species detected in eDNA tended to be maintained at a greater distance from the fishpond where they originated, compared to eRNA. Therefore, we conclude that environmental RNA is useful for fish ecosystem surveys in bays contaminated by fish feed because of the lower

content of RNA in the samples compared to DNA and because it is less easily detected and diffused in the environment.

Keywords: environmental RNA, false positives, fish feed, aquaculture, coastal area

Graphical Abstract



1. Introduction

Environmental DNA (eDNA) has emerged as a powerful tool for molecular ecological surveys, providing significant advantages over traditional survey methods in terms of cost, reproducibility, and comprehensiveness (Goldberg et al., 2016). This technique allows for continuous and high-quality monitoring of aquatic ecosystems, aligning well with global conservation goals such as Nature Positive set forth during COP15 (CONVENTION ON

BIOLOGICAL DIVERSITY (CBD), 2022; Tickner et al., 2020) as well as the Sustainable Development Goals (SDGs) related to conserving marine biodiversity. However, a critical challenge in the use of eDNA is the potential for false positives. This issue arises when DNA from deceased organisms, such as those found in wastewater, is mistakenly detected as evidence of living species in the surveyed environment (Inoue et al., 2023b; Xiong et al., 2024). Therefore, it is essential to consider scenarios of nucleic acid contamination when interpreting eDNA survey results.

Coastal bay areas, with their complex ecosystems and proximity to human activities, are particularly susceptible to various forms of contamination. These areas can be heavily influenced by chemical and pharmaceutical pollutants from domestic and industrial wastewater, recreational activities, and sediment runoff from construction (Micella et al., 2024; Vikas and Dwarakish, 2015). Bays are thus critical sites for monitoring chemical pollution impacts under the Nature Positive framework. However, this susceptibility also poses significant challenges for eDNA-based surveys. Fish nucleic acids may enter the bay through domestic waste (Inoue et al., 2023b), fish processing plant effluents, and aquaculture operations. Notably, fish feeds used in aquaculture can introduce extraneous fish nucleic acids into the environment, potentially compromising the accuracy of eDNA

surveys. Despite its importance, there has been little research on the impact of fish feed on false positives in eDNA surveys. Therefore, it is necessary to gather foundational information on the nucleic acids present in fish feed and analyze their distribution within a bay as a case study.

One promising approach to mitigate false positives in eDNA surveys is the use of environmental RNA (eRNA) (Cristescu, 2019; Yates et al., 2021). Unlike DNA, RNA degrades more rapidly, making it less likely to persist in the environment and thus reducing the risk of detecting non-living sources. Studies have shown that eRNA can yield higher positive detection rates (An et al., 2023; Li et al., 2024; Miyata et al., 2022, 2021) and that DNA concentrations in wastewater are significantly higher than RNA concentrations (Inoue et al., 2023b). Consequently, the amount of RNA from fish feed in both the feed itself and the bay water is expected to be lower than that of DNA, suggesting that eRNA could potentially enhance the accuracy of coastal ecosystem surveys by minimizing false positives.

In this study, we employed metabarcoding analysis of eDNA and eRNA to identify fish species present in aquaculture feeds and evaluate their potential impact on ecological

surveys in a heavily cultivated bay. Specifically, we aimed to (1) elucidate the species composition of fish nucleic acids in fish feeds for tuna (*Thunnus orientalis*), Red seabream (*Pagrus major*), and longtooth grouper (*Epinephelus bruneus*), (2) determine the distribution range of these feeds within a bay (Kushimoto fishing port) where those fishes are farmed, and (3) assess the efficacy of eRNA analysis in reducing false positives attributable to fish feed. Our findings provide crucial insights for improving the accuracy of eDNA-based ecological surveys in bays influenced by aquaculture activities.

2. Materials and Methods

2-1. Location and water sample collection

Kushimoto fishing port in Wakayama prefecture, Japan, a bay where various fishes are farmed was selected as a location of our study. Example of the farmed fishes are tuna (*Thunnus orientalis*), red seabream (*Pagrus major*), and longtooth grouper (*Epinephelus bruneus*). We collected water samples (n = 12: 1 sample/site × 10 sites + 2 blank controls) were collected, using van dorn water sampler (SANSYO Co., LTD Company, Tokyo, Japan), on August 2020 at five sites (Table 1) in culture preserves (3 L per site), one site in each of the four directions around the culture preserves (6 L per site), one sites in further north from the culture preserves (15 L per site), and two blank controls as inside and

outside of the culture preserve. The lowest amount of water that would yield sufficient DNA or RNA quantity was set. Purified water of 3 L/sample was filtered on site as the blank control.

Table 1. The conditions of water sampling.

Water sampling point	Coordinate	The distance from the culture preserve (m)	Water sample (L)
Origin point	N33◦27′ 38″, E135◦47′ 18″	-	Each 3
East point	N33◦27′ 39″, E135◦47′ 25″	150 ~ 200	6
West point	N33◦27′ 40″, E135◦47′ 11″	150 ~ 200	6
South point	N33◦27′ 32″, E135◦47′ 18″	150 ~ 200	6
North point	N33◦27′ 46″, E135◦47′ 21″	150 ~ 200	6
Further north point	N33◦27′ 53″, E135◦47′ 27″	450 ~ 500	15

Figure 1. Sampling points in the Kushimoto fishing port

Portrait acquisition date: May 11, 2018, Water collection date: August 25-27, 2020

2-1. Fish feeds used in this study.

Feeds used for farming for tuna, red seabream, and longtooth grouper at the Kushimoto Port were used to analyze the composition of fish nucleic acids (DNA and RNA) in those feeds (Table 1 and Figure 1). The feed for tuna (product name: Eden d6) containing 68% animal-based feed such as fishmeal and krill meal was purchased from FEED ONE CO., LTD. The feed for red seabream (product name: Madai EP super8) containing 50% animal-based feed such as fish meal was purchased from MARUBENI NISSHIN FEED

CO., LTD. The feed for longtooth grouper (product name: Kuetarou 20) containing 69% animal-based feed such as fishmeal and shrimp meal was purchased from HIGASHIMARU CO., LTD. Other ingredients in fish feeds were listed in Table 2.

Table 2. Fish feeds analyzed in the present study.

Product Name	Manufacturer	Target Fish Species	Ingredients
Eden d6	FEED ONE CO., LTD.	Tuna	68% animal-based feed (fishmeal, krill meal), 12% cereals (flour, starch), 20% others (fish oil, lecithin, calcium phosphate, betaine, yeast extract, lactobacillus plantarum, citric acid)
Madai EP super8	MARUBENI NISSHIN FEED CO., LTD.	Red Seabream	50% animal-based feed (fishmeal), 23% vegetable oil cake (corn gluten meal, soybean oil cake), 17% cereals (flour, starch), 10% others (refined fish oil)
Kuetarou 20	HIGASHIMARU CO., LTD.	Longtooth Grouper	69% animal-based feed (fishmeal, shrimp meal), 15% cereals (flour, starch), 5% soy (fermented lysine meal), 11% others (feed yeast, refined fish oil, calcium carbonate, cocoa bean shells, garlic powder, seaweed powder)

2-2. DNA/RNA metabarcoding analysis

Water and fish feeds sampling.

Water samples were filtered with Sterivex™ filter units (0.45 μm nominal pore size; Millipore, Billerica, MA, USA), and immediately after filtration, the filters were filled with 1.7-2.0 mL ice-cold RNA*later* (Thermo Fisher Scientific, Waltham, MA, USA). To prevent cross-contamination, new tubes and syringes were used for each filtration. The samples were stored at -80°C, and purified water was filtered on-site as a blank control. Fish feeds were in solid form, and one to several pellets of each were used (Eden d6: 356.1 mg, Madai EP super8: 632.1 mg, Kuetarou 20: 487.4 mg). These samples were crushed to the powder in 1.5 mL tube and then extracted DNA and RNA.

DNA and RNA extraction.

Total eDNA/eRNA (water) or DNA/RNA (fish feeds) co-extraction were performed using the RNeasy PowerSoil Total RNA Kit and the DNA Elution Accessory Kit (Qiagen, Hilden, Germany). The Sterivex cartridge, which water sample was filtered, in a 50 mL tube was centrifuged at 8,000-10,000 g for 3-10 min to remove RNA*later*. A total of 1.5 mL of lysis buffer mixture (composed of 1.25 mL PowerBead solution and 0.25 mL SR1 solution) was added to the cartridge through the inlet. The cartridge was incubated with gentle rotation at 60°C for 60 min. After incubation, the lysis buffer was collected by centrifuging the Sterivex cartridge in a 50 mL tube at 8,000 g for 1 min, and the

supernatant was transferred into a 15 mL PowerBead tube. This tube contained 1.25 mL of PowerBead solution, 0.8 mL of IRS solution, and 3.5 mL of phenol/chloroform/isoamyl alcohol. The mixture was then vortexed at maximum speed for 15 min. Subsequent steps followed the manufacturer's instructions, with an elution volume of 100 μL. DNA and RNA (fish feeds) were co-extracted according to the manufacturer's protocol with an elution volume of 100 μL. Following the co-extraction process, the DNA samples were purified using a NucleoSpin DNA Clean-up XS kit (MACHEREY-NAGEL, Düren, Germany). Prior to the cDNA synthesis, the RNA samples were treated twice with DNase, ultilizing an rDNase set and a NucleoSpin RNA Clean-up XS kit (MACHEREY-NAGEL, Düren, Germany). The synthesis of cDNA from single-stranded RNA was carried out with a PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's protocol. To evaluate potential cross-contamination during the processes of sample filtration, DNA/RNA co-extraction, and cDNA synthesis, both DNA and RNA were extracted concurrently from deionized water. All DNA/RNA and cDNA samples that were extracted were promptly stored at −80 °C.

Library preparation

Amplicon libraries were constructed using Takara Ex Taq Hot Start Version with fish

166 universal primer pairs for metabarcoding, MiFish-U and -E33 (Miya et al., 2015). The
167 first PCR involved a reaction mixture of 10 μL, consisting of 6.1 μL sterile distilled water,
168 1 μL 10 × PCR buffer, 0.8 μL dNTP mixture, 0.5 μL of each primer (10 μM), 0.1 μL
169 Takara EX Taq HS and 1 μL DNA or cDNA template. The thermal cycling conditions for
170 the first PCR consisted of an initial denaturation at 95 °C for 3 minutes, followed by 35
171 cycles at 98 °C for 20 seconds, 65 °C for 15 seconds and 72 °C for 20 seconds, concluding
172 with an extension at 72 °C for 5 minutes. The PCR amplification was repeated eight times
173 for each DNA and cDNA sample, and the products were purified using the AMPure XP
174 system (Beckman Coulter, Brea, CA, USA), which yielded a specific amplification of
175 approximately 380 bp fragments. For the second PCR, 0.5 μM primer pairs with MiSeq
176 adaptor sequences and 8 bp index sequences at both ends of the amplicons were employed.
177 The thermal cycling profile for this round included an initial step at 94°C for 2 minutes,
178 followed by 10-12 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30
179 seconds, concluding with an extension at 72°C for 5 minutes. The resulting PCR products
180 were purified using the AMPure XP system (Beckman Coulter) and equal volumes of the
181 purified amplicons were mixed. Finally, the library was sequenced using 2 × 300 bp
182 paired-end sequencing on an Illumina MiSeq platform with a MiSeq V3 Reagent Kit
183 (Illumina, San Diego, CA, USA).

Bioinformatics analysis of high-throughput sequencing data

The FASTX Barcoding Splitter, part of the FASTX-Toolkit v. 0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/; Hannon Laboratory, University of Cambridge, Cambridge, UK), was utilized to isolate the sequencing reads that perfectly matched the primers employed. The sequences of the primers, along with 120 bp from the 3′ end, were eliminated, and any chimeric sequences were filtered out using the QIIME 2 v. 2020.8 (https://qiime2.org/) package that incorporates DADA2 (https://benjjneb.github.io/dada2/dada-installation.html). This process generated a sequence table, which is a matrix containing amplicon sequence variants (ASVs) and the count of reads for each species per sample. For taxonomic classification, a BLASTN search v. 2.9.0 (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was conducted against the MitoFish database v. 3.53 (http://mitofish.aori.utokyo.ac.jp/; University of Tokyo, Tokyo, Japan) as well as the reference data relevant to the MiFish metabarcoding analysis.

2-3. Data analysis including statistics.

Composition of fish DNA/RNA in each fish feed and water samples was displayed using balloon plot, bar chart, heatmap, and Venn diagram, stacked bar chart. First, Venn diagram was employed to analyze fish numbers detected by eDNA/eRNA metabarcoding in each

fish feed and bar graph was used to visualize the amount of read numbers in metabarcoding analysis. Then, duplication of fish species detected in feed and water samples for each metabarcoding analysis. Finally, Relationship between distance from the fish tank (origin point) and the number of reads of water samples for each fish were evaluated using heatmap analysis.

3. Results

3-1. Composition of fish nucleic acids in each fish feed

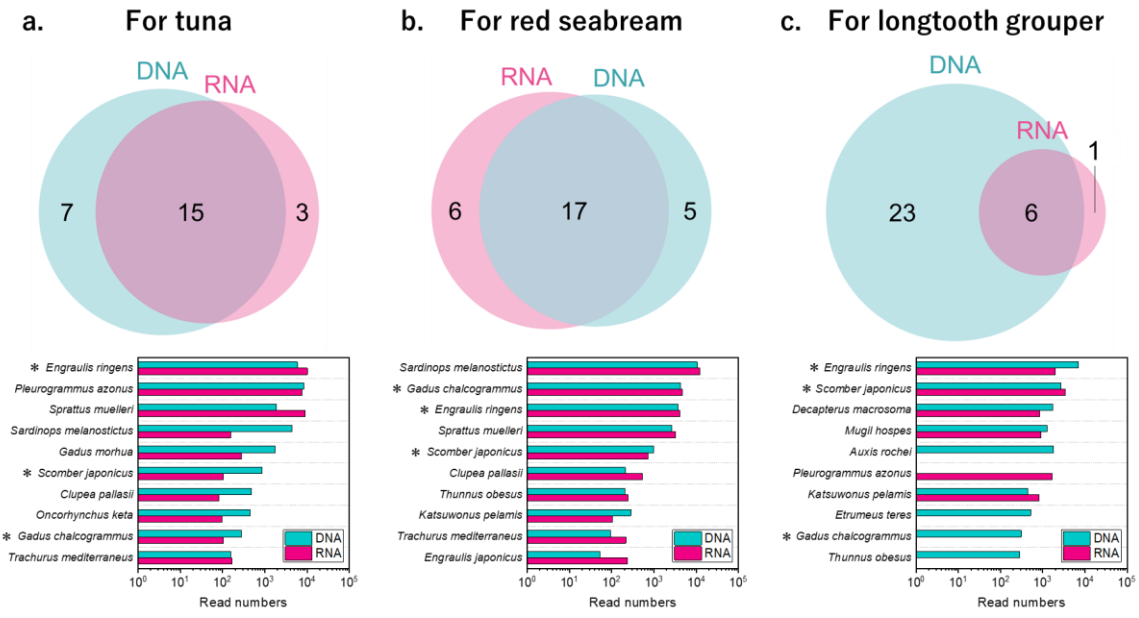


Figure 2. The number of detected fish species in fish feeds in DNA and RNA

Venn plots show the number of detected species and bar graphs show read numbers of eDNA and eRNA metabarcoding for top ten species detected, respectively, for (a) tuna, (b) red seabream, and (c) longtooth grouper. Cyan: eDNA; Magenta: eRNA. *: Detected

215 in all three feeds.

216

217 The feed instructions did not specify which animal based feed was derived from which
218 fish; Metabarcoding analysis revealed the fish species that comprised the diet. A total of
219 51 fish species (DNA: 46, RNA: 31) were detected, including 25 (DNA: 22, RNA: 18) in
220 the feed for tuna, 28 (DNA: 22, RNA: 23) in the feed for red seabream, and 30 (DNA: 29,
221 RNA: 7) in the bait for longtooth grouper. Although the detected species in eDNA is
222 equivalent to that in eRNA in the feed for red seabream, that in eDNA in the feeds for
223 tuna and longtooth grouper is less than eRNA in those. Focucing on the Top 10 species
224 with high read numbers in eDNA/eRNA metabarcoding analysis, *Engraulis ringens*
225 (Peruvian anchoveta), *Pleurogrammus azonus* (Okhotsk atka mackerel), *Sprattus*
226 *muelleri* (New Zealand sprat), *Sardinops melanostictus* (Pacific sardine), *Gadus morhua*
227 (Atlantic cod), *Scomber japonicus* (Pacific chub mackerel), *Clupea pallasii* (Pacific
228 herring), *Oncorhynchus keta* (Chum salmon), *Gadus chalcogrammus* (Alaska pollock),
229 and *Trachurus mediterraneus* (Mediterranean scad) in tuna feeds, *Sardinops*
230 *melanostictus* (Pacific sardine), *Gadus chalcogrammus* (Alaska pollock), *Engraulis*
231 *ringens* (Peruvian anchoveta), *Sprattus muelleri* (New Zealand sprat), *Scomber japonicus*
232 (Pacific chub mackerel), *Clupea pallasii* (Pacific herring), *Thunnus obesus* (Bigeye tuna),

Katsuwonus pelamis (Skipjack tuna), *Trachurus mediterraneus* (Mediterranean scad), and *Engraulis japonicus* (Japanese anchovy) in red seabream feeds, *Engraulis ringens* (Peruvian anchoveta), *Scomber japonicus* (Pacific chub mackerel), *Decapterus macrosoma* (*Shortfin scad*), *Mugil hospes* (Hospe Mullet), *Auxis rochei* (Bullet tuna), *Pleurogrammus azonus* (Okhotsk atka mackerel), *Katsuwonus pelamis* (Skipjack tuna), *Etrumeus teres* (Round herring), *Gadus chalcogrammus* (Alaska pollock), and *Thunnus obesus* (Bigeye tuna) in longtooth grouper feeds were detected. *Engraulis ringens* (Peruvian anchoveta), *Scomber japonicus* (Pacific chub mackerel), and *Gadus chalcogrammus* (Alaska pollock) were detected in all feeds.

3-2. Fish nucleic acids detected in the bay and the comparison with those in fish feeds

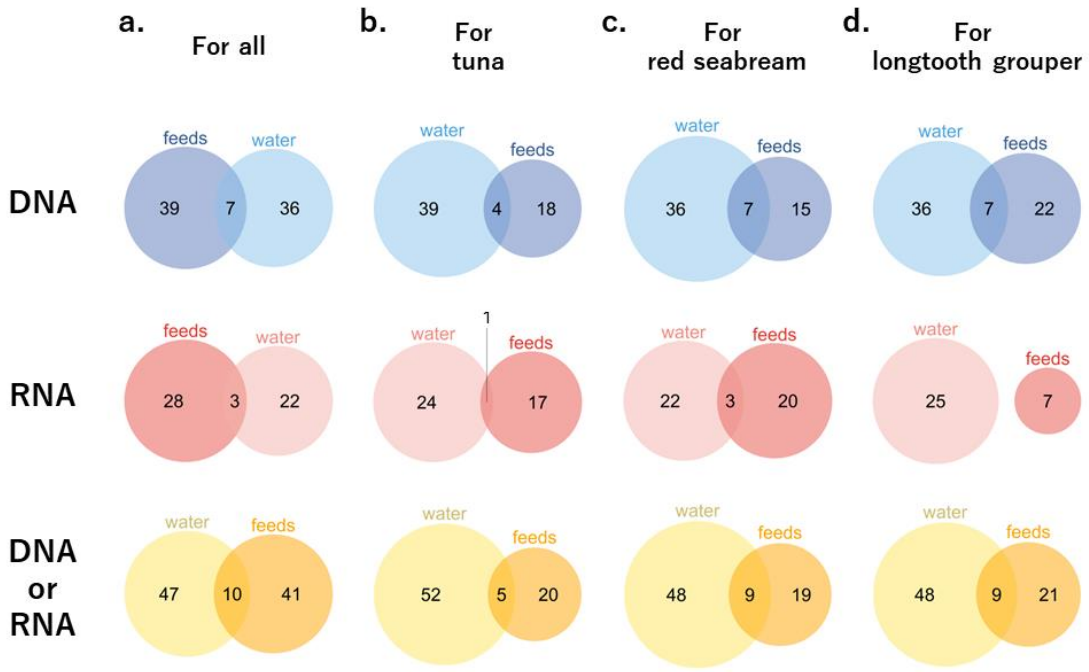


Figure 3. Comparison of detected fish species between fish feeds and water samples.

a) Venn diagram for whole samples, b) Venn diagram for the tuna feed, c) Venn diagram for the seabream feed, d) Venn diagram for the longtooth grouper feed

Table 3. Fish list common to fish feeds and water samples.

Species were also indicated in Figure 3 a. For all DNA and RNA.

Scientific name	English name
eDNA metabarcoding	
Pagrus major	Red seabream
Trachurus mediterraneus	Mediterranean scad
Scomber japonicus	Pacific chub mackerel
Sardinops melanostictus	Pacific sardine
Thunnus alalunga	Albacore

Seriola quinqueradiata	Japanese amberjack
Seriola dumerili	Greater amberjack
eRNA metabarcoding	
Engraulis japonicus	Japanese anchovy
Trachurus mediterraneus	Mediterranean scad
Thunnus obesus	Bigeye tuna

Metabarcoding analysis in the bay revealed a total of 57 fish species (DNA: 41, RNA: 24), of which 5 (DNA: 4, RNA: 1) were detected in the feed for tuna, 9 (DNA: 7, RNA: 3) in the feed for red seabream, and 9 (DNA: 7, RNA: 0) in the feed for longtooth grouper overlapped with the fish species detected in the feeds (Figure 3 and Table 3). Overlapped species of eDNA between water and feed samples were relatively more than those of eRNA. These species can cause high estimates of abundance when they are actually present and false positives when they are not.

3.3 Transportation effects of eDNA/eRNA in fish feeds.

We focused on the species detected in both food and water samples and analyzed the relationship between distance from the aquaculture facility using these diets and the number of water sample reads for target fishes. For eDNA, seven fish were detected at the starting point and three to five of the seven fish were detected at 175 and 475 m from

the origin point. For eRNA, three fish were detected at the origin point and no species were detected at 175 and 475 m. As the number of duplicated species and the number of reads for fish detected in food and water were high at 0 m compared to 475 m, these fish might be false positives. Although these fishes are common species and might habit in the sites, these fish species might have a low abundance and the false positives may be due to fish feeds. Since the number of detectable DNA/RNA fragments decreases with distance from the origin point, both DNA and RNA are less detectable at greater distances due to dilution and degradation by diffusion. However, environmental DNA was considered to be at greater risk of causing false positives over a wider geographic area.

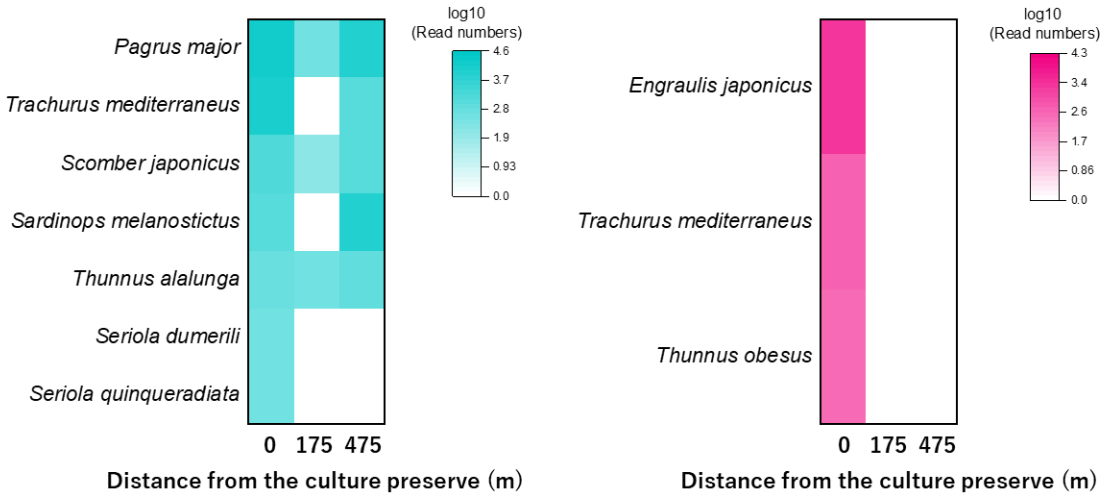


Figure 4. Relationship between distance from the fish tank (origin point) and the number of reads of water samples for each fish.

Heatmap of log10-transformed read numbers in (left; cyan) eDNA or (right; magenta) eRNA from water. Darker colors indicate higher values. White indicates absence. Columns correspond to each species. Distance from the culture preserve; 0 m: at the origin point, 175m: east, west, south, and north point, 475 m: further north point.

Discussion

In this study, we demonstrated that fish feed used in aquaculture contains various fish nucleic acids and is a significant source of false positives that threatens ecosystem studies around the Bay. Considering that the fish are fed several times a week with food equivalent to several percent of their body weight, the amount of food spilled is not negligible. Most of the food is expected to be fed to the fish, but even if only a few percent is left uneaten, the amount would be negligible. Also, since DNA is widely used to analyze the biological composition of at least the contents of the digestive tract (Alberdi et al., 2019), nucleic acids would be released as feces without degradation after being fed.

We hypothesized that the amount of RNA in fish feeds and the amount of RNA derived from fish diets in waters samples could be less than the amount of these DNAs, based on differences in ease of degradation in the environment (Jo et al., 2022; Kagzi et al., 2022;

Wood et al., 2020). Consistent with that prediction, the number of fish species in the diet detected by environmental DNA was less than or equal to those detected by environmental RNA. Of those species, the number of species detected in the environment was also less with eRNA than with eDNA. Although not strictly comparable, the number of reads detected in fish feeds in the metabarcoding analysis tended to be lower for eRNA than for eDNA, and similar tendency was observed in the environmental water samples. Based on the above, it is possible that environmental RNA may have been more degraded than environmental DNA during the fish feed production process or after entering the environment after feeding. Thus, environmental RNA metabarcoding has the potential to provide fewer false positives in coastal ecosystem studies.

We focused on environmental nucleic acid contamination (Inoue et al., 2023b) arising from the aquaculture industry in coastal areas and demonstrated the utility of environmental RNA-based surveys in this study. Although distancing from the point source of contamination is a necessary idea, a new approach should be considered to identify the false positive influx scenarios and the types of nucleic acids they contain. Inoue et al. (Inoue et al., 2023a) first reported a computational model that can estimate the amount and ratio of eDNA/eRNA in river water, and various other environmental

317 modeling methods related to eDNA have been developed (Andruszkiewicz et al., 2019;
318 Carraro et al., 2023, 2020; Fukaya et al., 2022). Moreover, environmental exposure
319 analysis model for chemical substances enabling connection bays and rivers have also
320 been reported (Miyake et al., 2014; Nishioka et al., 2019; Yamane et al., 2024). If
321 environmental modeling allows species-specific estimates of the levels of contaminating
322 nucleic acids, it will improve the accuracy of molecular ecological surveys using
323 eDNA/eRNA and accelerate their implementation in the construction of biodiversity
324 conservation strategies.
325
326 This study analyzed the usefulness of environmental RNA in reducing false positives in
327 terms of the number of fish detected and changes in the number of reads in metabarcoding
328 analysis; however, quantitative RT-PCR quantification is needed to determine exact
329 amount of nucleic acids in their feeds. Quantitative metabarcoding (Tsuji et al., 2022)
330 should be used to compare the amounts in the aquatic environment among sampling sites.
331 In addition, analysis of more fish feeds and accumulation of examples of analyses in bays
332 are needed.
333

In conclusion, by identifying the species composition of fish nucleic acids in the food of each species and analyzing the distribution of these foods in the bay, we tested whether eRNA analysis is effective in reducing false positives caused by fish food. The number of fish detected was higher for environmental DNA in both food and water samples. Therefore, the identification of nucleic acid species in fish diets is an important analysis item to be considered in ecological surveys, and environmental RNA was shown to be effective in reducing false positives. Regarding the effectiveness of environmental RNA in reducing false positives, further systematic analysis of composition in wastewater and aquaculture feed as possible sources should be conducted to elucidate the quantitative relationship with the species detected in the environment.

References

Alberdi, A., Aizpurua, O., Bohmann, K., Gopalakrishnan, S., Lynggaard, C., Nielsen, M., Gilbert, M.T.P., 2019. Promises and pitfalls of using high-throughput sequencing for diet analysis. Mol. Ecol. Resour. 19, 327–348. https://doi.org/10.1111/1755-0998.12960

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, 550. https://doi.org/10.3390/FISHES8110550/S1

Andruszkiewicz, E.A., Koseff, J.R., Fringer, O.B., Ouellette, N.T., Lowe, A.B., Edwards, C.A., Boehm, A.B., 2019. Modeling environmental DNA transport in the coastal ocean using Lagrangian particle tracking. Front. Mar. Sci. 6, 476826. https://doi.org/10.3389/FMARS.2019.00477/BIBTEX

Carraro, L., Blackman, R.C., Altermatt, F., 2023. Modelling environmental DNA transport in rivers reveals highly resolved spatio-temporal biodiversity patterns. Sci. Reports 2023 131 13, 1–14. https://doi.org/10.1038/s41598-023-35614-6

Carraro, L., Mächler, E., Wüthrich, R., Altermatt, F., 2020. Environmental DNA allows upscaling spatial patterns of biodiversity in freshwater ecosystems. Nat. Commun. 2020 111 11, 1–12. https://doi.org/10.1038/s41467-020-17337-8

CONVENTION ON BIOLOGICAL DIVERSITY (CBD), 2022. DECISION ADOPTED BY THE CONFERENCE OF THE PARTIES TO THE CONVENTION ON BIOLOGICAL DIVERSITY 15/29. Biodiversity and health The.

Cristescu, M.E., 2019. Can Environmental RNA Revolutionize Biodiversity Science? Trends Ecol. Evol. 34, 694–697. https://doi.org/10.1016/j.tree.2019.05.003

Fukaya, K., Kondo, N.I., Matsuzaki, S. ichiro S., Kadoya, T., 2022. Multispecies site

occupancy modelling and study design for spatially replicated environmental DNA metabarcoding. Methods Ecol. Evol. 13, 183–193. https://doi.org/10.1111/2041-210X.13732

Goldberg, C.S., Turner, C.R., Deiner, K., Klymus, K.E., Thomsen, P.F., Murphy, M.A., Spear, S.F., McKee, A., Oyler-McCance, S.J., Cornman, R.S., Laramie, M.B., Mahon, A.R., Lance, R.F., Pilliod, D.S., Strickler, K.M., Waits, L.P., Fremier, A.K., Takahara, T., Herder, J.E., Taberlet, P., 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods Ecol. Evol. 7, 1299–1307. https://doi.org/10.1111/2041-210X.12595

Inoue, Y., Miyata, K., Honda, H., 2023a. WO2023171758 FISH ECOLOGICAL SURVEY METHOD IN FRESHWATER ENVIRONMENT.

Inoue, Y., Miyata, K., Yamane, M., Honda, H., 2023b. Environmental Nucleic Acid Pollution: Characterization of Wastewater Generating False Positives in Molecular Ecological Surveys. ACS ES T Water 3, 756–764. https://doi.org/10.1021/acsestwater.2c00542

Jo, T., Tsuri, K., Hirohara, T., Yamanaka, H., Toshiaki Jo, C., 2022. Warm temperature and alkaline conditions accelerate environmental RNA degradation. Environ. DNA 00, 1–13. https://doi.org/10.1002/EDN3.334

Kagzi, K., Hechler, R.M., Fussmann, G.F., Cristescu, M.E., 2022. Environmental RNA degrades more rapidly than environmental DNA across a broad range of pH conditions . Mol. Ecol. Resour. 1–11. https://doi.org/10.1111/1755-0998.13655

Li, W., Jia, H., Zhang, H., 2024. Evaluating the effectiveness of the eRNA technique in monitoring fish biodiversity – A case study in the Qingdao offshore, China. Glob. Ecol. Conserv. 51, e02888. https://doi.org/10.1016/J.GECCO.2024.E02888

Micella, I., Kroeze, C., Bak, M.P., Strokal, M., 2024. Causes of coastal waters pollution with nutrients, chemicals and plastics worldwide. Mar. Pollut. Bull. 198, 115902. https://doi.org/10.1016/J.MARPOLBUL.2023.115902

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J.Y., Sato, K., Minamoto, T., Yamamoto, S., Yamanaka, H., Araki, H., Kondoh, M., Iwasaki, W., 2015. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes : detection of more than 230 subtropical marine species Subject Category : Subject Areas : R. Soc. Open Sci. 2, 150088. https://doi.org/10.1098/rsos.150088

Miyake, Y., Kobayashi, T., Kameya, T., Managaki, S., Amagai, T., Masunaga, S., 2014. Comparison study on observed and estimated concentrations of perfluorooctane sulfonate using a fate model in Tokyo Bay of Japan. J. Environ. Sci. Health. A. Tox. Hazard. Subst. Environ. Eng. 49, 770–776.

https://doi.org/10.1080/10934529.2014.882192

Miyata, K., Inoue, Y., Amano, Y., Nishioka, T., Nagaike, T., Kawaguchi, T., Morita, O., Yamane, M., Honda, H., 2022. Comparative environmental RNA and DNA metabarcoding analysis of river algae and arthropods for ecological surveys and water quality assessment. Sci. Rep. 12, 1–16. https://doi.org/10.1038/s41598-022-23888-1

Miyata, K., Inoue, Y., Amano, Y., Nishioka, T., Yamane, M., Kawaguchi, T., Morita, O., Honda, H., 2021. Fish environmental RNA enables precise ecological surveys with high positive predictivity. Ecol. Indic. 128, 107796. https://doi.org/10.1016/J.ECOLIND.2021.107796

Nishioka, T., Iwasaki, Y., Ishikawa, Y., Yamane, M., Morita, O., Honda, H., 2019. Validation of AIST-SHANEL Model Based on Spatiotemporally Extensive Monitoring Data of Linear Alkylbenzene Sulfonate in Japan: Toward a Better Strategy on Deriving Predicted Environmental Concentrations. Integr. Environ. Assess. Manag. 15. https://doi.org/10.1002/ieam.4167

Tickner, D., Opperman, J.J., Abell, R., Acreman, M., Arthington, A.H., Bunn, S.E., Cooke, S.J., Dalton, J., Darwall, W., Edwards, G., Harrison, I., Hughes, K., Jones, T., Leclère, D., Lynch, A.J., Leonard, P., McClain, M.E., Muruven, D., Olden,

J.D., Ormerod, S.J., Robinson, J., Tharme, R.E., Thieme, M., Tockner, K., Wright, M., Young, L., 2020. Bending the Curve of Global Freshwater Biodiversity Loss: An Emergency Recovery Plan. Bioscience 70, 330–342. https://doi.org/10.1093/BIOSCI/BIAA002

Tsuji, S., Inui, R., Nakao, R., Miyazono, S., Saito, M., Kono, T., Akamatsu, Y., 2022. Quantitative environmental DNA metabarcoding shows high potential as a novel approach to quantitatively assess fish community. Sci. Reports 2022 121 12, 1–11. https://doi.org/10.1038/s41598-022-25274-3

Vikas, M., Dwarakish, G.S., 2015. Coastal Pollution: A Review. Aquat. Procedia 4, 381–388. https://doi.org/10.1016/j.aqpro.2015.02.051

Wood, S.A., Biessy, L., Latchford, J.L., Zaiko, A., von Ammon, U., Audrezet, F., Cristescu, M.E., Pochon, X., 2020. Release and degradation of environmental DNA and RNA in a marine system. Sci. Total Environ. 704, 135314. https://doi.org/10.1016/j.scitotenv.2019.135314

Xiong, W., MacIsaac, H.J., Zhan, A., 2024. An overlooked source of false positives in eDNA-based biodiversity assessment and management. J. Environ. Manage. 358, 120949. https://doi.org/10.1016/J.JENVMAN.2024.120949

Yamane, M., Honda, H., Murata, M., Kawaguchi, T., Ishikawa, Y., 2024. Global-

SHANEL Asia model predicting chemical concentration in rivers with high spatio-temporal resolution, suitable for climate change scenarios. J. Environ. Manage. 370. https://doi.org/10.1016/J.JENVMAN.2024.122335

Yates, M.C., Derry, A.M., Cristescu, M.E., 2021. Environmental RNA: A Revolution in Ecological Resolution? Trends Ecol. Evol. 36, 601–609. https://doi.org/10.1016/J.TREE.2021.03.001

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