Title

Quantitative assessment of multiple fish species around artificial reefs using environmental DNA metabarcoding

Authors

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Abstract

Since the early 1970s, many artificial reefs (ARs) have been deployed in Japanese coastal waters to create fisheries grounds. Recently, researchers began to use environmental DNA (eDNA) methods for biodiversity monitoring of aquatic species. A metabarcoding approach using internal standard DNAs (i.e., quantitative MiSeq sequencing) makes it possible to monitor eDNA concentrations of multiple species simultaneously. This method can improve the efficiency of monitoring AR effects on fishes. Our study investigated distributions of marine fishes at ARs and surrounding stations in the open oceanographic environment of Tateyama Bay, central Japan, using quantitative MiSeq sequencing and echo sounder survey. Using the quantitative metabarcoding method, we found higher quantities of fish eDNAs at the ARs than at surrounding stations and different fish species compositions between them. Comparisons with eco sounder survey also showed positive correlations between fish eDNA concentration and echo intensity, which indicates a highly localized signal of eDNA at each sampling station. These results suggest that quantitative MiSeq sequencing is a promising technique to complement conventional methods to monitor distributions of multiple fish species.

38 **Introduction**

39 Artificial reefs (ARs) have been deployed worldwide to create fisheries grounds. Since the early
40 1970s, restrictions on overseas fishing have influenced high seas fisheries outside the exclusive
41 economic zones (EEZ), and this situation led the Japanese government to implement a new
42 fishery policy to develop and maintain coastal fisheries. In 1971, the Japanese government started
43 the first national project of maintenance and development of coastal fisheries and has continued to
44 implement similar projects, spending \$ 5.4 billion to deploy ARs throughout coastal waters.
45 Yamane¹ indicated that the total number of Japanese AR projects and proportion of shallow
46 fishing grounds with ARs in 1986 reached 6,443 and 9.3%, respectively. After establishing a
47 large number of ARs in Japanese waters, underwater visual census, surveys using fishery gear,
48 and echo sounder surveys have been used to examine fish aggregation and stock enhancement
49 effects of ARs in Japanese waters²⁻⁵. These survey methods have advantages and disadvantages.
50 An underwater visual census is effective for species identification, fish counts, and size
51 estimation, but it requires identification skills in situ and/or a long investigation time for large
52 areas. Surveys with bottom trawls and gillnets can obtain similar information on the fish to the
53 underwater visual census, but these surveys exploit fish individuals. Echo sounders can survey
54 large geographical areas quickly without fish collection, but with this method, it is difficult to
55 identify fish species.

56 Recently, researchers have begun to use environmental DNA (eDNA), DNAs derived from
57 environmental samples such as water, to investigate the distribution of aquatic organisms,
58 including marine fishes^{6,7}. In the case of macro-organisms, eDNA originates from metabolic
59 waste, damaged tissue, dead individuals, or spawning events⁸, and the eDNA contains
60 information about the species of the organisms that produced it⁷. This method does not need to
61 exploit a body or body tissues of target species and can detect target organisms noninvasively.
62 Also, this method requires less field time and is more likely to detect the presence of target species
63 than the other survey methods^{6,9}. Recently, the metabarcoding approach, using high-throughput
64 sequencing technologies (e.g., Illumina MiSeq) and universal primer sets, has been utilized to
65 reveal the fish diversity in a given area^{6,10}. MiFish, a universal polymerase chain reaction (PCR)
66 primer set, is especially popular for fish eDNA metabarcoding and has detected more fish species
67 than other competing primers¹¹.

68 Although the eDNA metabarcoding approach has become a cost- and labor-effective approach
69 for estimating aquatic biodiversity, it has one limitation, i.e., the quantity of eDNA cannot be
70 estimated from the number of eDNA sequence reads obtained by high-throughput sequencing
71 partly due to several experimental problems such as PCR inhibitions⁷. However, some studies
72 have proposed an approach using an internal standard DNA to overcome this limitation. Ushio et
73 al.⁷ tested whether additions of internal standard DNAs (i.e., known amounts of short DNA

74 fragments from fish species that have never been observed in a sampling area) to eDNA samples
 75 can create sample-specific regression lines between the numbers of sequence reads and DNA
 76 copies. Based on regression lines for each sample, they converted the sequence reads of DNAs
 77 from unknown fish species (i.e., non-standard fish species) to the number of fish DNA copies in
 78 each sample. This study showed that the estimated numbers of total and two dominant fish
 79 (Japanese anchovy and jack mackerel) DNA copies were significantly positively correlated with
 80 the numbers of DNA copies quantified by qPCR. Therefore, this method (i.e., quantitative MiSeq
 81 sequencing) can contribute to the quantitative assessment of multispecies fish eDNA. Some
 82 studies have shown that the eDNA quantity could be a proxy for the abundance or biomass of
 83 macro-organisms, not only in closed systems like tank and mesocosm¹², but also in marine
 84 ecosystems^{9,13}. The quantitative MiSeq sequencing with MiFish primer could be one of the most
 85 cost- and labor-effective approaches for the quantitative monitoring of multi species fish in
 86 marine and coastal ecosystems, including ARs.

87 Here, we investigated marine fish distributions around ARs in Tateyama Bay, central Japan,
 88 using eDNA metabarcoding, catch record of fishery, and eco sounder method. The quantitative
 89 MiSeq sequencing using internal standard DNAs and MiFish primers was applied to assess
 90 whether the distribution of multi fish species around ARs could be monitored based on the spatial
 91 variation in the estimated quantities of eDNA (i.e., identification of fish species and
 92 quantification of the number of fish eDNA copies simultaneously). Specifically, the following
 93 were tested: 1) whether fish communities detected by eDNA metabarcoding match with the catch
 94 record of the coastal fishery, 2) whether the DNA quantities of multiple fish species calculated by
 95 the quantitative MiSeq sequencing decrease with increasing distance from an AR, 3) whether the
 96 spatial variation of fish DNA quantities correlates to the relative distribution estimated by the
 97 acoustic survey.

98

99 **Materials and methods**

100 *Study site, field survey, and in situ filtration*

101 The field survey was performed in Tateyama Bay (34°60'N, 139°48'E), central Japan, in the
 102 proximity of the Kuroshio warm current facing the Pacific Ocean (Fig. 1). This area has many
 103 artificial reefs (ARs) created to improve fishing efficiency for fishers. Among the ARs, we
 104 focused on one high-rise steel AR (AR1), with a height of 30 m, where fish tended to aggregate
 105 (Fig. S1). Sampling stations were set up at the AR1 and at six linear distant points extending
 106 northeast and southwest. These stations were named E150, E500, E750, W150, W500, and W750,
 107 where “W” or “E” and the number of each station name represented northeast or southwest and
 108 distance in meters from the AR1, respectively (Table 1 and Fig. 1). Another station was set up at
 109 a second AR (AR2: 25 m height) 220 m from AR1 because we found AR2 by chance during the

110 survey (Table 1 and Fig. 1), and it might affect the eDNA concentration at other stations.

111 We conducted water sampling for eDNA analysis and performed an acoustic survey for
 112 estimating relative fish density using research vessel *Taka-maru* (Japan Fisheries Research and
 113 Education Agency: FRA) on May 23, 2018. We started the echo sounder survey at the southern
 114 part of the bay and continued it during the water sampling. Although the echo sounder survey
 115 could not differentiate between fish species, we collected this data to assess the association
 116 between the estimated concentration fish of eDNA and the echo intensity measured by the echo
 117 sounder. Water sampling began at E750, then continued along the transect line to E150, AR1,
 118 W150, W500, W750, before going back to AR2. At each sampling station, we collected 10 L of
 119 seawater from both the middle and bottom layers by one cast of two Niskin water samplers and
 120 measured vertical profiles of water temperature and salinity with a
 121 conductivity-temperature-depth sensor (RINKO profiler, JFE Advantech Co., Ltd.). Then, two
 122 2L samples were subsampled from the seawater samples and immediately filtered using a
 123 combination of Sterivex filter cartridges (nominal pore size = 0.45 μ m; Merck Millipore) through
 124 an aspirator (i.e., the two filters were subsets of a single water collection) in a laboratory on the
 125 research vessel. After filtration (approximately 15 min), an outlet port of the filter cartridge was
 126 sealed with an outlet luer cap, 1.5 ml RNAlater (ThermoFisher Scientific, Waltham, MA) was
 127 injected into the cartridge using a filtered pipette tip to prevent eDNA degradation, and an inlet
 128 port was also sealed with an inlet luer cap¹⁴. The Niskin water samplers were bleached before
 129 each water collection using a commercial bleach solution while filtering devices (i.e., filter
 130 funnels and measuring cups used for filtration) were bleached after every filtration. We filtered
 131 2L MilliQ water with a filter funnel and measuring cup for every two or three sites as a field
 132 negative control to test for possible contamination. The filter cartridges were placed in a freezer
 133 immediately after filtration until eDNA extraction. Disposable latex or nitrile gloves were worn
 134 during sampling and replaced between each sampling station.

135

136 *DNA extraction and purification*

137 Workspaces were sterilized prior to DNA extraction using 10% commercial bleach, and filter tip
 138 pipettes were used to safeguard against cross-contamination. Following the method developed by
 139 Miya et al.¹⁴, the eDNA was extracted and purified. Briefly, after removing RNAlater inside the
 140 cartridge using a centrifuge, proteinase-K solution was injected into the cartridge from the inlet
 141 port, and the port was re-capped with the inlet lure cap. The eDNA captured on the filter
 142 membrane was extracted by constant stirring of the cartridge at a speed of 20 rpm using a roller
 143 shaker placed in an incubator heated at 56°C for 20 min. The eDNA extracts were transferred to a
 144 2-ml tube from the inlet of the filter cartridges by centrifugation. The collected DNA was purified
 145 using a DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. After the

146 purification, DNA was eluted using 100 µl of the elution buffer (buffer AE). All DNA extracts
147 were frozen at -20°C until paired-end library preparation.

148

149 *Preparation of internal standard DNAs*

150 Five artificially designed and synthetic internal standard DNAs, which were similar but not
151 identical to the region of any existing fish mitochondrial 12S rRNA, were included in the library
152 preparation process to estimate the number of fish DNA copies (i.e., quantitative MiSeq
153 sequencing)^{7,15}. They were designed to have the MiFish primer-binding regions as those of
154 known existing fishes and to have the conserved regions in the insert region. Variable regions in
155 the insert region were replaced with random bases so that no known existing fish sequences had
156 the same sequences as the standard sequences. The standard DNA size distribution of the library
157 was estimated using an Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA), and the
158 concentration of double-stranded DNA of the library was quantified using a Qubit dsDNA HS
159 assay kit and a Qubit fluorometer (ThermoFisher Scientific, Waltham, MA, USA). Based on the
160 quantification values obtained using the Qubit fluorometer, the copy number of the standard
161 DNAs was adjusted as follows: Std. A (100 copies/µl), Std. B (50 copies/µl), Std. C (25
162 copies/µl), Std. D (12.5 copies/µl) and Std. E (2.5 copies/µl). Then, these standard DNAs were
163 mixed.

164

165 *Paired-end library preparation*

166 Four PCR - level negative controls (i.e., each two with and without internal standard DNAs) were
167 employed for MiSeq run to monitor contamination during the experiments. The first-round PCR
168 (1st PCR) was carried out with a 12-µl reaction volume containing 6.0 µl of 2 × KAPA HiFi
169 HotStart ReadyMix (KAPA Biosystems, Wilmington, WA, USA), 0.7 µl of each primer (5 µM),
170 2.6 µl of sterilized distilled H₂O, 1.0 µl of standard DNA mix and 1.0 µl of template. Note that the
171 standard DNA mix was included for each sample. The final concentration of each primer was 0.3
172 µM. We used a mixture of the following four PCR primers modified from original MiFish
173 primers¹⁶: MiFish-U-forward (5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT
174 NNN NNG TCG GTA AAA CTC GTG CCA GC) and MiFish-U-reverse (5'-GTG ACT GGA
175 GTT CAG ACG TGT GCT CTT CCG ATC TNN NNN CAT AGT GGG GTA TCT AAT CCC
176 AGT TTG-3'), MiFish-E-forward (5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA
177 TCT NNN NNG TTG GTA AAT CTC GTG CCA GC-3'), and MiFish-E-reverse (5'-GTG ACT
178 GGA GTT CAG ACG TGT GCT CTT CCG ATC TNN NNN CAT AGT GGG GTA TCT AAT
179 CCT AGT TTG-3'). These primer pairs co-amplify a hypervariable region of the fish
180 mitochondrial 12S rRNA gene (around 172 bp; hereafter called MiFish sequence) and append
181 primer-binding sites (5' ends of the sequences before six Ns) for sequencing at both ends of the

182 amplicon. The five random bases were used to enhance cluster separation on the flow cells during
183 initial base call calibrations on the MiSeq platform. The thermal cycle profile after an initial 3 min
184 denaturation at 95°C was as follows (35 cycles): denaturation at 98°C for 20 s; annealing at 65°C
185 for 15 s; and extension at 72°C for 15 s, with a final extension at the same temperature for 5 min.
186 Eight replications were performed for the 1st PCR, and the replicates were pooled to minimize the
187 PCR dropouts. The 1st PCR products from the eight tubes were pooled in a single 1.5-ml tube.
188 Then, we sent the 1st PCR products to IDEA consultants, Inc. to outsource the following MiSeq
189 sequencing processes. The pooled products were purified and size-selected for 200–400bp using a
190 SPRIselect (Beckman Coulter inc.) to remove dimers and monomers following the
191 manufacturer’s protocol.

192 The second-round PCR (2nd PCR) was carried out with a 24 µl reaction volume containing 12
193 µl of 2 × KAPA HiFi HotStart ReadyMix, 2.8 µl of each primer (5 µM), 4.4 µl of sterilized
194 distilled H₂O, and 2.0 µl of template. We used the following two primers to append the
195 dual-index sequences (8 nucleotides indicated by Xs) and flowcell-binding sites for the MiSeq
196 platform (5′ ends of the sequences before eight Xs): 2nd-PCR-forward (5′-AAT GAT ACG GCG
197 ACC ACC GAG ATC TAC ACX XXX XXX XAC ACT CTT TCC CTA CAC GAC GCT CTT
198 CCG ATC T-3′); and 2nd- PCR-reverse (5′-CAA GCA GAA GAC GGC ATA CGA GAT XXX
199 XXX XXG TGA CTG GAG TTC AGA CGT GTG CTC TTC CGA TCT-3′). The thermal cycle
200 profile after an initial 3 min denaturation at 95°C was as follows (12 cycles): denaturation at 98°C
201 for 20 s; combined annealing and extension at 72°C for 15 s, with a final extension at 72°C for 5
202 min. The concentration of each second PCR product was measured by quantitative PCR using TB
203 Green Fast qPCR Mix (Takara inc.). Each sample was diluted to a fixed concentration and
204 combined (i.e., one pooled 2nd PCR product that included all samples). The pooled 2nd PCR
205 product was size-selected to approximately 370 bp using BluePippin (SAGE SCIENCE). The
206 size-selected library was purified using the Agencourt AMPure XP beads, adjusted to 4 nM by
207 quantitative PCR using TB Green Fast qPCR Mix (Takara inc.), and sequenced on the MiSeq
208 platform using a MiSeq v2 Reagent Kit (2 × 150 bp).

209

210 *Data preprocessing and taxonomic assignment*

211 The raw MiSeq data were converted into FASTQ files using the bcl2fastq program provided by
212 Illumina (bcl2fastq v2.18). The FASTQ files were then demultiplexed using the command
213 implemented in Claident¹⁷. We adopted this process rather than using FASTQ files demultiplexed
214 by the Illumina MiSeq default program in order to remove sequences whose 8-mer index
215 positions included nucleotides with low-quality scores (i.e., Q-score < 30).

216 The processed reads were subjected to a BLASTN search against the full NCBI database. If the
217 sequence similarity between queries and the top BLASTN hit was < 98.5% and the sequence

length was less than ≤ 150 bp, the unique sequence was not subjected to the following analyses. After BLASTN searches, assembled sequences assigned to the same species were clustered, and we considered the clustered sequences as operational taxonomic units (OTUs). To remove possible contaminants, we removed the OTUs whose sequence reads were < 0.05 % of the total reads as a noise for each sample.

Catch record of coastal fishery

To confirm the feasibility of MiFish metabarcoding, we compared the fish community of MiFish metabarcoding with a catch record of a net set near the study site (Fig.1). The detection rate was defined as the proportion of species detected by metabarcoding compared to the species collected by the net. Because our eDNA survey was conducted in May 2018, we used catch-record data from the same period (May 2018).

Estimation of DNA copy numbers

According to Ushio et al.⁷ and Ushio¹⁵, the procedure to estimate DNA copy numbers consisted of two parts: (i) linear regression analysis to examine the relationship between sequence reads and the copy numbers of the internal standard DNAs for each sample and (ii) the conversion of sequence reads of non-standard fish DNAs to estimate the copy numbers using the result of the linear regression for each sample.

Linear regressions were used to examine how many sequence reads were generated from one fish DNA copy through the library preparation process. Note that a linear regression analysis between sequence reads and standard DNAs was performed for each sample, and the intercept was set as zero. The regression equation was: MiSeq sequence reads = regression slope $\times$ the number of standard DNA copies [μ l]. The number of linear regressions performed was thirty-three (=the number of fish DNA samples + field negative controls with standard DNAs), and thus thirty-three regression slopes were estimated in total.

The sequence reads of non-standard fish DNAs were converted to copy numbers using sample-specific regression slopes estimated by the above regression analysis. The number of non-standard fish DNA copies was estimated by dividing the number of MiSeq sequence reads by a sample-specific regression slope (i.e., the number of DNA copies = MiSeq sequence reads/regression slope). A previous study demonstrated that these procedures provide a reasonable estimation of DNA copy numbers using high-throughput sequencing⁷.

Acoustic survey of fish school using echo sounder

We estimated relative fish density using a quantitative echo sounder following the acoustic survey of Yamamoto et al.⁹. We used the echo sounder, KFC-6000 (Sonic Co. Tokyo, Japan), with a

transducer (frequency, 38 kHz; beam type, split-beam; beam width, approximately 7°; pulse duration, 0.3 ms; ping rate, 0.4 s). The transducer was set at the bottom of the research vessel at a depth of 2.1 m to avoid cavitation bubbles generated by the research vessel. The acoustic devices were operated during the entire survey cruise, and all signals were recorded. The average ship speed was ~10 knots between sampling stations, although the ship slowed down when approaching a sampling station and completely stopped when water samples were being collected.

We eliminated noise from the obtained echo intensity data using Echoview ver. 11.0 (Echo-view Software Pty. Ltd., Tasmania, Australia). Signals between the sea bottom and 1.0 m above the sea bottom were eliminated to avoid possible confounding with the acoustic dead zone. In addition, the signals of Niskin water samplers and RINKO profiler were also eliminated. Finally, we eliminated the signals of bubbles generated by the movement of the screw propeller. After eliminating these noises, we re-obtained echo intensity data to assess the association with eDNA concentration. Note that the obtained echo intensity data would include echo signals from a variety of fish species.

Data analyses

We performed three types of univariate analysis using generalized linear models (GLMs). First, we used likelihood ratio tests to assess variation in the eDNA copy numbers of total fish, demersal fish, pelagic fish, and five dominant OTUs, the number of OTUs, as well as echo intensity among sampling stations and between vertical positions of water sampling. When the effects of sampling stations were significant, the Tukey-HSD test was used to determine the significant differences between sampling stations. The second analysis assessed the number of OTUs and eDNA copy numbers of total, demersal, pelagic, and five dominant OTUs in relation to distance from the AR1, vertical position of water samples (i.e., middle or bottom layers, represented as 'depth'), and the interaction between the distance and vertical position. Although our sampling stations contained two ARs, we focused on AR1 in the second analysis because more fish aggregated around AR1 and transport of eDNAs from AR1 may have more impact than those from AR2. The third analysis examined the relationships between eDNA copy numbers (each eDNA copy number of total fish, demersal fish, pelagic fish, or five dominant OTUs described below) and echo intensity (S_v). Because echo intensity can correlate to distance from the AR1, the second and third analyses were separately performed. Based on Fishbase¹⁸, each fish OTU was classified into demersal and pelagic species. For the analyses, we used the Poisson distribution with a log-link function for OTU number because it was count data, while a gamma distribution with a log-link was used for eDNA copy numbers of total fish, pelagic fish, demersal fish, and *Scomber* spp. (one dominant OTU) because these values are continuous and greater than zero. A mixture of binomial

and gamma distributions, which is often called a “gamma hurdle model,” was also used as in previous studies^{19,20} for eDNA copy numbers of remaining dominant OTUs because these values are continuous and contain zero values. Bayesian information criterion (BIC) was used to select the correct model among candidate models for the second and third analyses²¹.

We also performed multivariate analyses to determine differences in the fish community structures between sampling stations/vertical positions. Community structures of each sampling were visualized using non-metric dimensional scaling (NMDS) based on the Bray–Curtis dissimilarity index of the copy numbers of each OTUs. Similarity tests between two variables (sampling stations and vertical positions) were conducted using nonparametric multivariate analysis of variance (NPMANOVA). All statistical analyses were performed using RStudio version 1.1.423 (R version 3.6.1) with several packages. The packages used were: (a) glmmTMB²² for regression models; (b) multcomp²³ for Tukey-HSD test; (c) MuMIn²⁴ for BIC model selection, (d) vegan²⁵ for multivariate analysis; (e) ggplot2²⁶ and (f) ggpubr²⁷ for figure creation.

Results

Sequence reads

The MiSeq paired-end sequencing (2×150 bp) of the 34 libraries for this study (containing 32 PCR replicates, one negative field control, and one PCR-level control), together with 142 libraries from another study (total number of libraries = 176), yielded a total of 15.98 million reads, with 95.3% base calls containing Phred quality scores of ≥ 30.0 (Q30; error rate = 0.1% or base call accuracy = 99.9%).

For this study, we obtained 4,862,616 MiSeq reads, of which 3,528,543 were high-quality, merged reads. Number of non-standard fish Miseq reads were 2,153,777 out of the 3,528,543 (61.0%). In the MiSeq run, 18.8%–98.6% of sequence reads were from non-standard fish DNAs of field samples, and 0% was from those of a field negative control. The final list included 95 OTUs distributed across 49 families and 83 genera (Table S1).

Comparison of eDNA metabarcoding with fish catch record

The fish catch record of a set net indicated that 289 thousand kg of total fish biomass belonging to 41 classifications (39 species and two classifications of closely related species) was caught in May 2018. The catch biomass was highly variable between the classifications. For example, 255 thousands kg of *Scomber japonicus* was recorded, whereas *Fistularia petimba* was 0.2 kg only (Table S2). Of the 41 classifications caught by the set net, eDNA metabarcoding detected 25 species. Three classifications of the 41 classifications were not recorded at the species level in the list of the fish catch of the set net, while the metabarcoding cannot assign OTUs at the species

level for the *Scomber japonicus* and *S. australasicus* because they are so closely related. Thus, excluding these five species, our metabarcoding eventually detected 58.3% (= 21/36) of the fishes caught by the set net. Among the five dominant OTUs detected by MiFish metabarcoding described below (*B. splendens*, *P. trilineatum*, *Scomber* spp., *P. major*, and *T. japonicus*), four OTUs except *B. splendens* appeared in the list of the fish catch.

Relationship between the copy numbers and sequence reads of the standard DNA

The relationships between the sequence reads and copy numbers of standard DNAs were examined by linear regression analysis (Fig. 2). Within each sample, the sequence reads linearly and positively correlated with the copy numbers of standard DNAs (Fig. 2a). R^2 values of the regression lines ranged from 0.71 to 0.99, and more than 75% of the samples had values over 0.9 (Fig. 2b). These results suggested that the number of sequence reads was generally proportional to the number of copies of DNA initially added to the first PCR reaction within a single sample. The slopes of the linear regressions varied depending on the sample, suggesting that the amplification efficiency or removal amounts of DNAs during the purification of PCR products were dependent on the individual sample. These sample-specific slopes were used to convert sequence reads to the copy numbers of fish OTUs. Based on the estimated copy numbers of each OTU, five dominant OTUs were *Beryx splendens*, *Parapristipoma trilineatum*, *Scomber* spp. (*S. japonicus* or *S. australasicus*), *Pargus major*, and *Trachurus japonicus* (Fig. 1b–f).

Levels of contaminations were assessed using the field negative control. DNA copy numbers detected in the field negative control ($n = 1$) were 0% of mean DNA copy numbers of field-positive samples, suggesting that there was no amount of contamination during the library preparation process.

Site and vertical variation in fish eDNA quantities, the number of OTUs, and echo intensity

The estimated copy numbers of fish OTUs were summed to estimate the total, demersal, and pelagic fish eDNA quantities. The average eDNA copy numbers of total fish, demersal fish, and pelagic fish were 64.3, 44.6, and 19.7 copies/ml water, respectively, while those of five dominant OTUs, *B. splendens*, *P. trilineatum*, *Scomber* spp., *P. major*, and *T. japonicus*, were 20.3, 13.8, 12.9, 5.5, and 3.2 copies/ml water, respectively. Likelihood ratio tests showed that the eDNA copy numbers significantly varied among sampling stations for total, demersal, pelagic fishes, *P. trilineatum*, *Scomber* spp., *P. major*, and *T. japonicus*, whereas no significant variation was detected for *B. splendens* (Table S3). Tukey-HSD tests indicated that significantly higher total and demersal fish eDNA copy numbers were observed at AR1 than at W150–750 and E 500–750, while the pelagic fish eDNA copy number was higher at AR1 than at E750 (Fig. S1). Also, the total, demersal, and pelagic fish eDNA quantities were significantly higher at AR2 than at E750.

For dominant fish OTUs, *P. trilineatum*, *P. major*, and *T. japonicus* eDNA copies were significantly higher at AR1 than at other stations, while *Scomber* spp. eDNA quantities were higher at AR1 and AR2 than at E750. Echo intensity (S_v) ranged from 4.53×10^{-8} to 1.38×10^{-6} , and a likelihood ratio test indicated significant variation in echo intensity among the stations. Like the fish eDNA quantities, the echo intensity was higher at AR1 than at the surrounding stations. Meanwhile, the number of OTUs detected at each station ranged from 9 to 27, with a mean of 16 OTUs, and there was no significant variation among the stations (Table S3).

Aside from echo intensity, likelihood ratio tests indicated that all the types of fish eDNA quantities and number of OTUs were significantly higher in the bottom samples than in the middle ones (Table S3 and Fig. S2).

Effects of distance from an artificial reef on fish eDNA quantities and the number of OTUs

Distance from the AR1 and vertical position were included in the Gamma component of the minimum BIC model for total fish, demersal fish, pelagic fish, and *Scomber* spp. eDNA quantities (Table 2). Estimated parameters indicated that these eDNA quantities decreased with increasing distance from the AR1 and the eDNA quantities were lower in the middle samples than in the bottom ones. For *Parapristipoma trilineatum*, *Pargus major*, and *Trachurus japonicus* eDNA quantities, distance from the AR1, vertical position, and their interaction were included in the Gamma component of the minimum BIC model. Estimated parameters indicated that these eDNA quantities decreased with increasing distance from AR1, but the intercepts and slopes of the relationship with distance differed between the layers. The eDNA quantities of *P. major* were lower in the middle layer at 0 m from AR1, while those of *P. trilineatum* and *T. japonicus* were higher in the middle samples at 0 m from AR1 (Fig. 3). Slopes for *P. trilineatum*, and *T. japonicus* eDNA quantities were steeper in the middle layer, while that for *P. major* eDNA was steeper in the bottom layer. Only the vertical position was included in the Gamma component of the minimum BIC model for the number of OTUs and *Beryx splendens* eDNA quantity, and these values were higher in the bottom layer than in the middle layer (Table 2 and Fig. 3).

Relationships between fish eDNA quantities and echo intensity

Based on the BIC values, echo intensity was selected as an explanatory variable for total fish, demersal fish, pelagic fish, *B. splendens*, *P. trilineatum*, *Scomber* spp., and *T. japonicus* eDNA, but not for *P. major*. The former eDNA quantities had positive relationships with echo intensity, and echo intensity had high predictive power for these eDNA quantities (Fig. 4 and Table 3).

Fish community structure

There was dissimilarity in the NMDS in fish community structure between the AR and other

surrounding stations (Fig. 5, NMDS stress = 0.096). PERMANOVA detected significant differences in community structure between the ARs (AR1 and AR2) and surrounding stations (E and W) as well as between the middle and bottom sampling layers (ARs vs surrounding stations: $F = 3.040$, $p = 0.005$; Middle vs bottom: $F = 4.542$, $p = 0.002$).

Discussion

Field studies have begun to use an eDNA approach for monitoring fish density or diversity in coastal ecosystems^{6,7,9,10}, but, as far as we know, there have been no studies using this method to assess artificial reef (AR) effects on marine fishes. We found that eDNA metabarcoding efficiently detected the fish assemblages around ARs of Tateyama Bay. MiFish metabarcoding detected 95 OTUs from 32 eDNA samples collected within 8 h on May 23, 2018 (Table S1), while 41 classifications (39 species and two classifications of closely related species) were recorded in the fish catch of the net set near the study site in May 2018 (Table S2). Of the 36 species recorded in the fish catch over a month, MiFish metabarcoding detected 58.3% of species (i.e., 21 species/36 species). This detection rate of metabarcoding relative to the conventional approach was within the range of previous MiFish metabarcoding studies (45–70%)^{6,28,29}. Four dominant OTUs in terms of eDNA quantities (*P. trilineatum* *Scomber* spp., *P. major*, and *T. japonicus*) were also a higher proportion of fish catch of the set net (Table S1). Meanwhile, MiFish metabarcoding detected 70 fish OTUs that were not collected by the set net. This difference in the detected fish community between the MiFish metabarcoding and set net may be partly because the set net could not collect small-sized fish, and non-targeted fishes (e.g., Myctophidae and Gobiidae) were not recorded in the fish catch. These results indicated that eDNA metabarcoding approach had a high and reasonable resolution for examining a fish community in the study site.

We observed linear relationships between the number of sequence reads and the quantities of internal standard DNAs. This result suggested that the sequence reads were proportional to the copy numbers of DNA within a single sample, indicating that the conversion of sequence reads using a sample-specific regression slope would provide an estimation of DNA copy numbers in the DNA extract (i.e., estimated DNA copy number = sequence reads/sample-specific regression slope). This finding is consistent with previous studies showing that this procedure provides a reasonable estimate of copy numbers of multispecies eDNAs^{7,15}. Meanwhile, the quantitative Miseq also has limitations; i.e., this method does not take account of the variation in amplification efficiency among species. Therefore, we have to caution against comparisons of DNA copy numbers between different species.

This study efficiently estimated the eDNA quantities of multiple fish species around an AR. Quantitative MiSeq sequencing found that the eDNA quantities of total, demersal, pelagic fishes,

434 and four dominant OTUs, except *B. splendens*, were higher at AR1 than adjacent sites. In
435 addition, all eDNA quantities, except *B. splendens*, sharply decreased with increasing distance
436 from the AR. In particular, those of *P. trilineatum*, *P. major*, and *T. japonicus* clearly decreased
437 even 150 m from AR1 (e.g., W150 and E150). These results are consistent with previous studies
438 showing a higher density of fishes in ARs than reference sites^{2,30,31}. In fact, four dominant OTUs,
439 *P. trilineatum*, *Scomber* spp., *P. major*, and *T. japonicus*, were known to appear and aggregate in
440 ARs^{2,31–33}. Only *B. splendens* was not reported to be dependent on ARs, although it is highly
441 targeted by fisheries and usually caught by vertical longline at midnight in a deeper zone of this
442 region (depth > 200 m, personal communication, M. Sato). A review paper of the biology of *B.*
443 *splendens* indicated that the fisheries grounds of this species around the mouth of Tokyo Bay are
444 100–500 m in depth, but their juveniles are sometimes collected in a shallower area (<100 m)³⁴.
445 Although we cannot exclude the possibility that eDNA from adult *B. splendens* could be detected
446 in this study, its higher eDNA quantities in the stations of 70–80 m depth (e.g., AR2, AR1 and
447 E150) would be likely to reflect the presence of its juveniles. In future studies, combining eDNA
448 analysis with other methods such as observation by underwater drones or GoPro, which can
449 determine their body length, would clarify the details of ontogenetic habitat shift for *B. splendens*.

450 We expected the number of OTUs would be higher at ARs than the surrounding stations, but
451 did not observe such a pattern. In the following year's survey, we found a larger number of fishes
452 around AR1 than surrounding stations in a GoPro video (personal observation, N. Inoue, M. Sato,
453 N. Furuichi), which contradicted our eDNA results. We suspect that higher eDNA concentrations
454 of dominant OTUs at ARs consumed a large portion of the sequence reads, and species with low
455 eDNA concentrations were not detected by MiFish metabarcoding in this study.

456 MiFish metabarcoding also showed different fish species composition between the AR and
457 surrounding stations based on NMDS and PERMANOVA. The eDNA quantities of fishes,
458 including dominant OTUs (pelagic fishes: *Scomber* spp., *Etrumeus teres*, *Sardinops*
459 *melanostictus*; demersal fishes: *B. splendens*, *P. trilineatum*, *P. major*, *Sardinops melanostictus*,
460 *Sacura margaritacea*, *Thamnaconus modestus*) were more abundant in ARs, while those of some
461 demersal fishes such as flathead (*Platycephalus* sp.2), Asian sheephead wrasse (*Semicossyphus*
462 *reticulatus*), barred knifejaw (*Oplegnathus fasciatus*), and seabream (*Dentex tumifrons* or *D.*
463 *cardinalis*) were higher in the surrounding stations. Different fish responses to AR structures may
464 result in differences in the fish community compositions between the AR and surrounding
465 stations.

466 Previous studies have found positive relationships between the density of target species and
467 eDNA concentrations in semi-closed marine systems like estuaries^{9,13}; our results found such
468 associations in an open marine system. We found eDNA quantities of total, demersal, pelagic
469 fishes, and four dominant species, except *P. major*, positively correlated with the echo intensity

470 measured by echo sounder. Our study site faces toward the open sea (i.e., Pacific ocean), and the
471 flow was stronger than in a previous study (5.0–25.0 cm/s in our study and 0.4–13.5 cm/s in a
472 semi-closed bay at Maizuru, Japan)³⁵. Therefore, the flow is likely to transport more eDNAs from
473 sampling stations in this study, but we could also obtain a strongly localized eDNA signal at
474 sampling stations. One exception is the eDNA quantity of *P. major*, which did not correlate with
475 echo intensity. This finding was probably due to elimination of the echo signals around the sea
476 bottom, where this species is mainly found¹⁸. Overall, our result indicates that eDNA quantities
477 reflect spatial distributions of marine fish in an open coastal environment.

478 We found the eDNA quantities and species number were higher in the bottom sampling layers
479 than in the middle layers. Multivariate analyses, including NMDS and PERMANOVA, also
480 showed different fish compositions between the bottom and middle layers. Previous eDNA
481 studies have also indicated variations in eDNA quantities, the number of species, and community
482 compositions between different sampling depths^{6,9,36,37}. Some of these studies using eDNA
483 methods suggested that water column stratification and the different species' responses to it cause
484 the observed different composition between sampling depths^{36,37}. Murakami et al.³⁵ also discussed
485 that the vertical distribution of eDNA depends on both the depth of the source organisms and the
486 behavior of eDNA, with the latter dependent on its chemical condition and physical factors in the
487 environment. Although water column stratification between the middle and bottom layers was not
488 observed in our study (Fig. S3), we consider that the preference of detected fish species for
489 bottom habitats or the behavior of eDNA contributed to the higher eDNA quantities and OTU
490 number in the bottom layer in this study.

491 Overall, our results demonstrate the effectiveness of quantitative MiSeq sequencing to assess
492 AR effects on multiple fish species. We found higher quantities of fish eDNA at AR stations than
493 at surrounding stations and positive correlations between these quantities and eco intensity, which
494 indicates a highly localized eDNA signal in an oceanographically dynamic environment. This
495 spatial resolution indicates that quantitative MiSeq sequencing can improve techniques to
496 monitor the distribution of multiple fish species. Another method, called 'quantitative sequencing
497 (qSeq),' has also been developed for simultaneous quantifications of eDNA concentrations of
498 multiple species³⁸. This method adopts a stochastic labeling approach with random-tag sequences
499 and can also quantify eDNA concentrations of fish species using the MiFish primer set³⁹. The
500 quantitative metabarcoding methods (i.e., the MiSeq sequencing and qSeq) can complement
501 conventional methods, especially in an environment with areas where the water is too deep to
502 adopt underwater visual census (>40m depth) or in no-take marine reserves where exploiting fish
503 is prohibited.

504

505 **Data availability**

506 DDBJ accession numbers of the DNA sequences analyzed in the present study are DRA011864
507 (Submission ID), PRJDB11389 (BioProject ID) and SAMD00298249–SAMD00298281
508 (BioSample ID).

509

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517

Author Contributions

519 M. S., R. N., and N. F. designed the study and conducted field surveys. T. I. and N. I. analyzed the
520 data of echo sounder survey. M. S. and N. I. performed molecular experiments, and M. U.
521 prepared the internal standards for eDNA quantification. M. S. and N. I. analyzed fish community
522 dataset extracted from results of MiSeq sequencing. M. S. wrote the first draft of the manuscript
523 and other authors commented on the manuscript.

524

Additional information

526 **Supplementary information** accompanies this paper.

527 **Competing Interests:** The authors declare no competing interests.

528

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630 Figures and Tables

631
632
633 Figure 1 (a) Location of sampling stations, cruise track, and a set net in Tateyama Bay. Gray areas
634 indicate landmasses, a gray bold line indicates cruise track, and gray thin lines indicate depth
635 contours with an interval of 20 m. Pictures of the dominant species, (b) splendid alfonsino
636 (*Beryx splendens*), (c) chicken grunt (*Parapristipoma trilineatum*), (d) chub mackerel (*Scomber*
637 *japonicus*), (e) red seabream (*Pagrus major*), and (f) jack mackerel (*Trachurus japonicus*).
638 Photograph credits: (b) Fumie Yamaguchi, (c, d, f) Yutaro Kawano, and (e) Masaaki Sato.
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642 Figure 2 The relationships between sequence reads and the copy numbers of standard DNAs
643 according to Ushio et al.⁷. (a) The relationships between sequence reads and the copy numbers of
644 standard DNAs with the maximum, median, and minimum slopes. (b) Distribution of R^2 values of
645 the regression lines.

646

Figure 3 The relationships between distance from AR1 as well as number of OTUs and eDNA copies of total fish, demersal fish, pelagic fish, *Beryx splendens*, *Parapristipoma trilineatum*, *Scomber* spp., *Pagrus major*, and *Trachurus japonicus*. The black line and the gray shaded area represent a median value and 95% confidence interval, respectively, as determined from the generalized linear model. Red and turquoise blue circles indicate values in the bottom and middle sampling layers.

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657 Figure 4 The relationships between eco intensity and eDNA copies of total fish, demersal fish,
658 pelagic fish, *Beryx splendens*, *Parapristipoma trilineatum*, *Scomber* spp., *Pagrus major*, and
659 *Trachurus japonicus*. The black line and the gray shaded area represent a median value and the
660 95% confidence interval, respectively, as determined from the generalized linear model. Red and
661 turquoise blue circles indicate values in the bottom and middle sampling layers.

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Figure 5 Nonmetric multidimensional scaling (NMDS) for fish communities detected at sampling stations. NMDS stress was 0.096.

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671 Table 1 Details of the eight sampling stations for fish eDNA survey on May 23, 2018

Station	Vertical position	Latitude (N)	Longitude (E)	Depth (m)	eDNA sampling time	eDNA sampling depth (m)	Water temperature (°C)	Salinity
E750	Middle	35.0020	139.7949	94	9:23	47	17.90	34.51
	Bottom				9:34	92	17.26	34.51
E500	Middle	34.9999	139.7937	86	10:08	43	18.50	34.49
	Bottom				10:13	84	17.45	34.49
E150	Middle	34.9971	139.7920	76	10:58	38	18.61	34.49
	Bottom				11:03	74	17.60	34.50
AR1	Middle	34.9957	139.7917	73	11:46	35	18.65	34.49
	Bottom				11:40	71	18.24	34.52
W150	Middle	34.9947	139.7906	71	12:35	35	19.43	34.46
	Bottom				12:39	69	18.67	34.50
W500	Middle	34.9928	139.7876	71	13:13	35	19.41	34.47
	Bottom				13:17	69	19.20	34.48
W750	Middle	34.9916	139.7853	67	13:52	32	19.93	34.44
	Bottom				13:56	65	19.43	34.48
AR2	Middle	34.9938	139.7911	65	14:33	30	19.96	34.48
	Bottom				14:26	63	19.13	34.47

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Table 2. Parameters estimated from an BIC minimum model based on generalized liner modelling.

	Estimate	SE	Five dominant species	Estimate ^b	SE ^b	Estimate ^c	SE ^c
Number of OTUs			<i>Beryx splendens</i> eDNA ^a				
Intercept	2.87	0.06	Intercept	3.02	0.36	-3.30	1.02
Distance			log(Distance+0.1)				
Vertical position (middle)	-0.25	0.10	Vertical position (middle)	-1.64	0.50		
Distance : Vertical position			log(Distance+0.1) : Vertical position (middle)				
Total fish eDNA			<i>Parapristipoma trilineatum</i> eDNA ^a				
Intercept	5.42	0.41	Intercept	3.01	0.57	-3.30	1.02
log(Distance+0.1)	-0.30	0.06	log(Distance+0.1)	-0.23	0.10		
Vertical position (middle)	-1.25	0.36	Vertical position (middle)	0.55	0.84		
log(Distance+0.1) : Vertical position (middle)			log(Distance+0.1) : Vertical position (middle)	-0.41	0.15		
Demersal fish eDNA			<i>Scomber</i> spp. eDNA				
Intercept	5.08	0.41	Intercept	-1.86	0.41		
log(Distance+0.1)	-0.34	0.06	log(Distance+0.1)	-0.15	0.07		
Vertical position (middle)	-1.17	0.35	Vertical position (middle)	-1.86	0.41		
log(Distance+0.1) : Vertical position (middle)			log(Distance+0.1) : Vertical position (middle)				
Pelagic fish eDNA			<i>Pargus major</i> eDNA ^a				
Intercept	4.10	0.45	Intercept	3.21	0.47	-6.16	3.60
log(Distance+0.1)	-0.23	0.06	log(Distance+0.1)	-0.39	0.08	0.62	0.56
Vertical position (middle)	-1.34	0.39	Vertical position (middle)	-5.19	0.69	3.18	1.24
log(Distance+0.1) : Vertical position (middle)			log(Distance+0.1) : Vertical position (middle)	0.46	0.13		
			<i>Trachurus japonicus</i> eDNA ^a				
			Intercept	1.83	0.52	-3.30	1.02
			log(Distance+0.1)	-0.24	0.09		
			Vertical position (middle)	0.14	0.72		
			log(Distance+0.1) : Vertical position (middle)	-0.31	0.13		

^aGamma hurdle model was used. ^bAn estimate and error of Gamma component when using Gamma or Gamma hurdle model. ^cAn estimate and error of binomial component when using Gamma hurdle model.

Table 3. Summary results of Gamma or Gamma hurdle models (Null model: model with intercept only; Model with eco intensity: minimum BIC model which includes eco intensity as an explanatory variable) for each response variable of eDNA copy numbers based on automated model selection.

	Intercept ^b	log(eco intensity) ^b	Intercept ^c	log(eco intensity) ^c	BIC	R ²
Total fish eDNA						
Null model	4.16				325.60	0.00
Model with eco intensity	18.97	0.94			315.40	0.41
Demersal fish eDNA						
Null model	3.80				299.20	0.00
Model with eco intensity	19.88	1.02			288.20	0.45
Pelagic fish eDNA						
Null model	2.98				251.90	0.00
Model with eco intensity	15.50	0.79			244.70	0.33
<i>Beryx splendens</i> eDNA ^a						
Null model	3.04		-3.43		235.70	0.00
Model with eco intensity	18.84	1.00	-3.43		230.70	0.44
<i>Parapristipoma trilineatum</i> eDNA ^a						
Null model	2.66		-3.43		202.20	0.00
Model with eco intensity	20.60	1.17	-3.43		184.80	0.52
<i>Scomber</i> spp. eDNA						
Null model	2.56				212.90	0.00
Model with eco intensity	16.32	0.87			207.60	0.37
<i>Pargus major</i> eDNA ^a						
Null model	2.04		-0.94		150.60	0.00
Model with eco intensity	13.99	0.75	-0.94		153.60	0.30
<i>Trachurus japonicus</i> eDNA ^a						
Null model	1.22		-2.71		118.50	0.00
Model with eco intensity	15.62	0.93	-2.71		130.70	0.40

^aGamma hurdle model was used. ^bAn estimate and error of Gamma component when using Gamma hurdle model. ^cAn estimate and error of binomial component when using Gamma hurdle model.