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# Vertical distribution of harmful algae in the sediment of Uranouchi Inlet by metabarcoding

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Uranouchi Inlet, situated on the Pacific coast of southwestern Japan, has been a highly enclosed inlet known for yellowtail farming since 1959. Since the 1980s, harmful algal blooms (HABs) have repeatedly occurred, resulting in mass mortality of fish and shellfish. In the sediment at the inlet, the resulting cysts of the HAB species may be preserved, which reflects the history of HAB events. However, the vertical distributions of HAB species in sediment have not been elucidated. In this study, core sediment samples were analyzed by metabarcoding. The dating of each sample was cited from previous study dating the same samples. The findings revealed the presence of eleven HAB species, with notable shifts from approximately 1977–1988. The timing of the shifts corresponded to that of the development of aquaculture and the resulting eutrophication. Vertical core metabarcoding provides footprints of how HAB species composition may be influenced by anthropogenic environmental changes.

## KEYWORDS

metabarcoding, 18S rDNA, eutrophication, anthropogenic impact, HABs, core sample

## 1 Introduction

Uranouchi Inlet, located on the Pacific coast of southwestern Japan, is a highly enclosed inlet with a narrow bay mouth (Yamaguchi et al., 2018). These characteristics enabled the start of yellowtail farming in 1959. Since then, fish farming has continued in the inlet, with nearly 3,500 tons of farmed fish (mainly yellowtail or red sea bream) produced in 2018 (Ministry of Agriculture, Forestry and Fisheries, 2018; Takahashi et al., 2021a). Harmful algal blooms (HABs) have repeatedly occurred in the inlet since the 1980s, resulting in mass mortality of fish and shellfish (Takahashi et al., 2021a).

Some HAB-causative species are known to form cysts, resulting in the accumulation of these cysts in sediments. These cysts germinate when the surrounding environment is

suitable for growth (Borsato et al., 2023; Brosnahan et al., 2020; Ellegaard and Ribeiro, 2018; Zingone and Oksfeldt Enevoldsen, 2000). It has been reported that some cysts in bottom sediments collected by a core sampler and found to be almost a century old could still germinate (Feifel et al., 2012, 2015; Härnström et al., 2011; Lundholm et al., 2011; Miyazono et al., 2012), which enables us to speculate on the history of HABs caused by cyst-forming species by identifying the species of cysts in bottom sediments. However, species identification of HAB cysts is difficult because of the lack of understanding of their morphological characteristics in some cases (Hallegraeff et al., 2003; Thoha et al., 2019; Yamaguchi et al., 1995). Recently, DNA-based species identification by metabarcoding via high-throughput sequencers has been performed to detect HAB-causative species in surface sediment samples because of the fast, work-saving, and comprehensive identification of multiple species (Dzhembekova et al., 2018; Liu et al., 2023; Wang et al., 2022a, 2022b). Studies that attempt to identify HAB species by collecting surface sediment samples at shallow depths (from 0 to 15 cm of collected surface sediments) have been performed to determine the HAB species that have been present recently in the nearby collection area. However, several studies have focused on the detection of HAB-causative species collected from sediment core samples that are more than 15 cm depths (Armbrrecht et al., 2024; Boere et al., 2011; Coolen et al., 2013; Siano et al., 2021). Among them, few studies have focused on how marine eukaryotic HAB communities have been influenced by anthropogenic activities, such as heavy metal pollution and agricultural pollution (Siano et al., 2021), climate change (Boere et al., 2009) and nutrient runoff from rivers (Coolen et al., 2013). Under these circumstances, there are no studies on the long-term history of the transition of HAB-causative species due to eutrophication in coastal areas caused by aquaculture. In this study, we aimed to clarify the vertical distribution of cyst-forming HAB species in sediment core samples from Uranouchi Inlet by metabarcoding, where fish farming has been continuously performed since the 1960s and HAB events have repeatedly occurred, and discuss the possibility that environmental changes caused by fish farming have contributed to changes in the community compositions of HAB species.

## 2 Method

The sediment core sample (0–57 cm depth) was collected as described by Takahashi et al. (2021a) at Menokuso Station in Uranouchi Inlet, Kochi, Japan (33.25.346N, 133.23.522E), on August 22, 2016 (Supplementary Figure 1). The sediment core was sliced into 3-cm layers each with a thread saw, and the nineteen layered samples were named URA01 (0–3 cm) to URA19 (54–57 cm), which were obtained as described previously by Takahashi et al. (2021a). To avoid contamination between each sample, only the center of each sediment sample was collected and peripheral sediment was removed by washing with sterile seawater. Prior to DNA extraction, the samples were stored in the dark at -80°C to prevent degradation of the genomic DNA of cysts of HAB species in

the sediment. Radiometric dating of the nineteen samples (Figure 1) was conducted with Pb-210 and Cs-137 by Takahashi et al. (2021a). The result of estimated year of each sample by Takahashi et al. (2021a) was shown in Figure 1.

The processes required for MiSeq sequencing, such as DNA extraction and MiSeq library preparation, were essentially performed following the methods described by Funaki et al. (2022). DNA was extracted from 250 mg of raw sediment taken from each layer of the core sample in duplicate via a DNeasy® PowerSoil® Kit according to the manufacturer's protocol (QIAGEN, Hilden, Germany). The eukaryotic universal V8–V9 primer set was used to amplify the V8–V9 region of the 18S rDNA (approximately 350 bp). The primers used were forward primer V8F+adapter 5'-TCGTCGGCAGCGTCAGATGTGTAT AAGAGACAG+ATAACAGGTCTGTGATGCCCT-3' and reverse primer 1510R+adapter 5'-GTCTCGTGGGCTCGGAGATGT GTATAAGAGACAG+CCTTCYGCAGGTTACCTAC-3' (Funaki et al., 2022). PCR was conducted using the primers and the extracted DNA from core samples as templates, and the amplified products (approximately 400 bp) were purified via the method described in Funaki et al. (2022). Equimolar quantities of the purified amplicons were pooled and subjected to 2 × 250-bp sequencing (MiSeq Reagent Nano Kit v2, Illumina) on an inhouse MiSeq platform (Illumina). The raw data (fastq file) of both the forward and reverse sequences obtained from MiSeq were deposited in the DDBJ Sequence Read Archive under BioProject number PRJDB17880 (DRR Run number: DRR543997–DRR544015).

After MiSeq paired-end sequencing (2 × 250 bp), raw sequences were trimmed in Mothur ver. 1.40.3 (Schloss et al., 2009) on Galaxy (Afgan et al., 2018) and Mothur ver. 1.36.1 (Schloss et al., 2009) in the laboratory based on the bioinformatics method described by Funaki et al. (2022). After trimming the sequences, only the sequences expected for the 18S rDNA V8–V9 region were retrieved, and singleton sequences were also removed based on the method of Funaki et al. (2022).

For HAB species identification of unique sequences, a BLAST (basic local alignment search tool) 2.7.1+ search (Camacho et al., 2009) was conducted via the Protist Ribosomal Reference database ver. 5.0.1 (PR<sup>2</sup>, Guillou et al., 2013). After a BLAST search against the reference database described above, only sequences showing more than 97% similarity to those of HAB species and more than 281-bp query coverage were selected for further analysis. To understand the full picture of eukaryotes obtained by MiSeq, the total number of reads in all samples of each taxon group from Supergroup to Division was calculated, as well as the relative number of reads in each of the 19 samples for each taxon group. Since this study focused on HAB species in sediment samples, unique sequences associated with HAB genera and species listed in the IOCUNESCO Taxonomic Reference List of Harmful Micro Algae (Lundholm et al., 2009) were retained. In addition, the genus *Skeletonema*, which is known to cause nori bleaching in Japan (Hori et al., 2019; Imai et al., 2006; 2021, Minamiura and Yamaguchi, 2019; Sakamoto et al., 2021) and caused fish kill events by *Skeletonema costatum* in British Columbia, Canada (Kent et al., 1995) was also treated as a HAB genus.

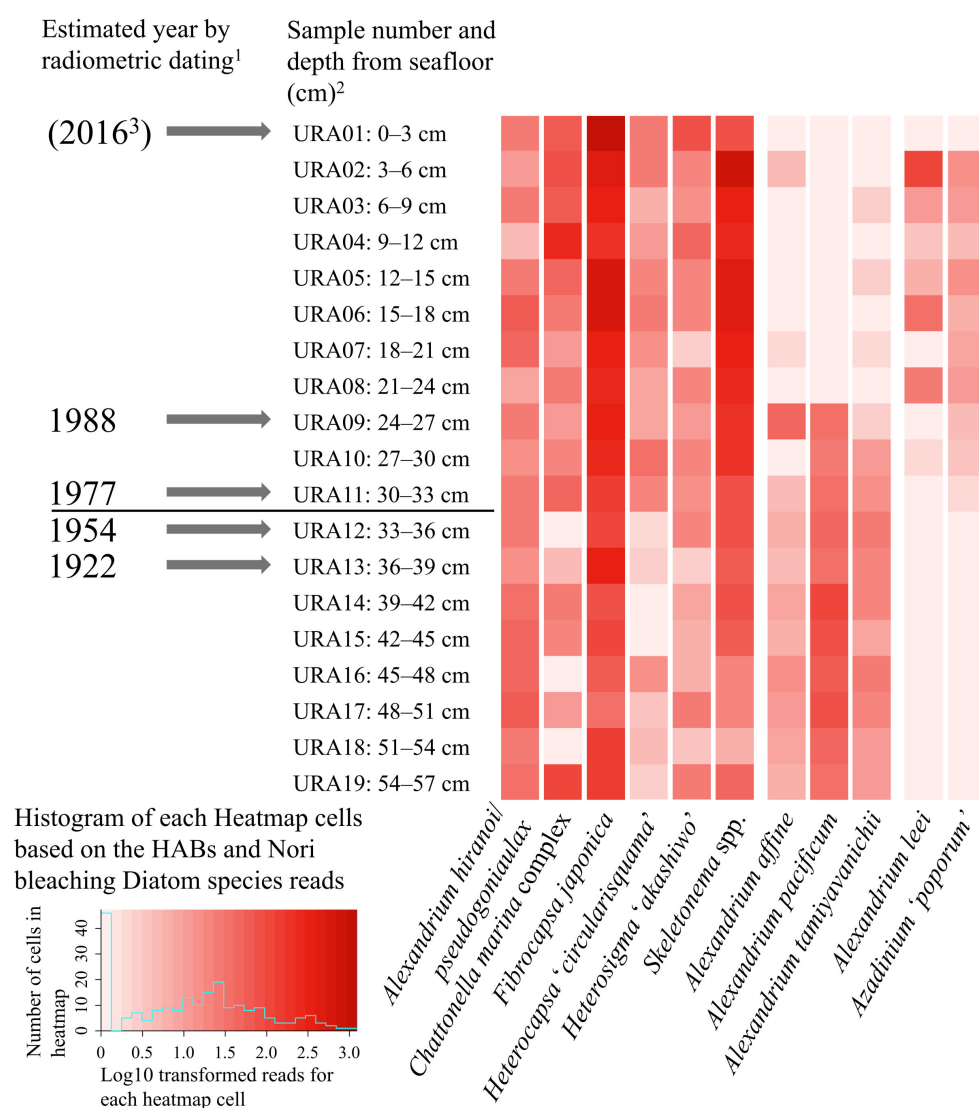


FIGURE 1

Heatmaps and histogram based on the log<sub>10</sub> transformed read numbers of HAB species and nori bleaching diatoms contained in the sediment samples from Uranouchi Inlet, Kochi, Japan. The solid line in the age column on the left side of the figure indicates the start of the aquaculture in Uranouchi Inlet. <sup>1</sup>: Radiometric dating using Pb-210 and Cs-137 of sediment samples was determined by Takahashi et al. (2021a). <sup>2</sup>: URA01–03, URA04–13 and URA14–19 were surface mixed layers, layers determined by radiometric dating, and layers out of the dating range, respectively. <sup>3</sup>: The core sample was collected from Uranouchi inlet in 2016.

To prevent misidentification of HAB species with unique sequences at the genus or species level, maximum likelihood (ML) molecular phylogenetic trees were constructed using unique sequences and reference sequences belonging to each genus obtained from GenBank and PR<sup>2</sup>. Multiple alignments, best model selection and ML molecular phylogenetic trees were constructed via the methods described by Funaki et al. (2022). If the unique sequence appeared in the same clade as the reference HAB species, it was assigned to the same HAB species. However, if the reference sequences of multiple HAB species were mixed in the same clade, misidentification was avoided by listing multiple species together.

To investigate the vertical distribution of HAB species detected in the sediment samples, a heatmap with hierarchical clustering was

generated on the basis of the read numbers of each HAB species in each sediment sample. The detailed heatmap analysis method followed the same approach as that in Funaki et al. (2022) and was performed in R 4.3.1 (R Core Team, 2023) and RStudio 2023.06.0 + 421 (RStudio Team, 2023).

To discuss the occurrence of HAB species detected by metabarcoding, the occurrence species of major HAB species and their cells measured by light microscopy when fishery damage occurred in Uranouchi Inlet from 1984 to 2017 was provided by the Kochi Prefectural Fisheries Experiment Station. Simultaneously, water quality survey results (NH<sub>4</sub>-N, NO<sub>2</sub>-N, NO<sub>3</sub>-N, PO<sub>4</sub>-P, DIN-N, DON-N, DOP-P, T-N and T-P) from 1980 to 2020 surveyed every 2 to 5 years in Mitsumatsu station near Menokuso Station in Uranouchi Inlet, where the core sample used in this study was

collected, was provided by Kochi Prefectural Fisheries Experiment Station and Fisheries Research Institute, Japan Fisheries Research and Education Agency.

## 3 Results

### 3.1 Overview of metabarcoding results

A total of 561,941 raw sequences were obtained from MiSeq paired-end sequencing, and all the raw sequences were generated as contig sequences (Supplementary Table 1). The sequences filtered and trimmed via Mothur were 179,827 unique sequences, and 465,118 reads were obtained. Among the sequences, 164,789 unique sequences and a total of 433,983 reads of the 18S rDNA V8–V9 region were obtained. After removal of chimeras and ‘pre.cluster’, 35,897 unique sequences and a total of 332,895 reads were passed through filtering. After singleton sequences were removed, 10,224 unique sequences and a total of 307,222 reads were obtained (Supplementary Table 1).

Eight supergroups of Eukaryota were identified from metabarcoding sequences using BLAST search with PR<sup>2</sup> database (Supplementary Table 2 and Supplementary Figure 2). The total read number identified by the BLAST search in the 19 sediment samples was 234,173 reads, with the highest number of 139,129 reads for Alveolata in the TSAR (Supplementary Table 2). Total reads of the supergroups and divisions derived from the 19 sediment samples were varied among 5,124 reads at URA18 to 17,703 reads at URA02 (Supplementary Table 3). Supergroup and division showing the most abundant reads was TSAR and Alveolata, respectively, in URA01–URA14 and URA19 samples (ranged from 33.79% at URA19 to 79.83% at URA09, Supplementary Table 4 and Supplementary Figure 2). In contrast, Obazoa and Opisthokonta were the most dominant supergroup and division in URA15–URA18 (ranged from 33.86% at URA15 to 42.59% at URA17), respectively.

### 3.2 Molecular phylogenetic position of each identified HAB species

Ten HAB species belonging to the genera *Alexandrium*, *Azadinium*, *Chattonella*, *Fibrocapsa*, *Heterocapsa*, and *Heterosigma* were identified in this study (Table 1). In the case of the genus *Skeletonema*, the total numbers of unique sequences and reads of the genus were also shown in Table 1 because not only *Skeletonema costatum* but also many species of the genus are considered to cause nori bleaching in Japan. The HAB species *Aureococcus anophagefferens*, *Azadinium dexteroporum* and *A. spinosum*, *Dictyocha fibula*, *Karenia mikimotoi*, *Pseudo-nitzschia delicatissima*, *P. multiseriata*, *P. pseudodelicatissima*, and *P. pungens* were also detected. However, the total read numbers of those HAB species were less than fifty which was difficult to examine for vertical

distribution in the sediment samples, so those species were excluded from the subsequent analysis.

In the molecular phylogenetic tree of the genus *Alexandrium*, unique sequences of the genus *Alexandrium* obtained this study were separated into five major clades (Supplementary Figure 3). Four clades contained sequences of *A. affine*, *A. leei*, *A. pacificum* (group IV) and *A. tamiyavanichii*, whereas the remaining clade contained two species (*A. hiranoi* and *A. pseudogonyaulax*) in the molecular phylogenetic tree. *A. pacificum* (Group IV) and *A. tamiyavanichii* have been reported as paralytic shellfish toxin (PST) producers (*A. pacificum*: Brown et al., 2021; Li et al., 2011; Wang et al., 2005, *A. tamiyavanichii*: Hashimoto et al., 2002; Lim et al., 2006; Sagara et al., 2010, Table 1). *A. affine* and *A. leei* have been reported to be associated with fish mortality (*A. affine*: Nguyen-Ngoc, 2004, *A. leei*: Shikata et al., 2020; Tang et al., 2007, Table 1). *A. hiranoi* and *A. pseudogonyaulax* produce goniodomin A, which is an antifungal polyether macrolide that inhibits actin reorganization and angiogenesis (Murakami et al., 1988, 1998; Triki et al., 2016, Table 1).

Regarding the toxic species of the genus *Azadinium*, three unique sequences belonging to the clade *A. poporum*/*A. dalianense* were obtained (Supplementary Figure 4). Since *A. dalianense* has not been found and *A. poporum* has recently been found in Japanese coastal waters via many water samples collected from various locations in Japan (Takahashi et al., 2021b), these unique sequences may be those of *A. ‘poporum’*, known as an azaspiracid producer (Krock et al., 2015; Luo et al., 2017, 2018; Tillmann et al., 2011, 2018, Table 1). The azaspiracid is known to cause symptoms similar to diarrhetic shellfish poisoning, including nausea, vomiting, abdominal pain, and severe diarrhea symptoms in humans who ingest mussels or scallops contaminated with azaspiracids (Twiner et al., 2008).

The unique sequences belonging to the *Chattonella marina* complex (*C. marina* var. *antiqua*, *C. marina* var. *marina*, *C. marina* var. *ovata* and *C. minima*) were detected (Supplementary Figure 5). Several strains of the *C. marina* complex produce reactive oxygen species (ROS), which may affect the gills of fish during red tide outbreaks and cause fish mortality (Shikata et al., 2021, Table 1). Regarding phylogeny, the *C. marina* complex detected in the sediment sample may also have the potential for ROS production because sequences of the productive ROS strains and those of the *C. marina* complex detected in this study appeared in the same clade on the tree (Supplementary Figure 5). The unique sequences assigned to *Fibrocapsa japonica* were detected (Supplementary Figure 5). Fish mortality caused by *F. japonica* has been reported (Iwasaki, 1971; Landsberg, 2002, Table 1), and some studies have reported that this species produces ROS and hemolysins, which may cause cell clogging in the gills (de Boer et al., 2009, 2012). The unique sequence of *Heterosigma ‘akashiwo’*, which has been reported as a HAB that causes fish mortality, was detected (Table 1 and Supplementary Figure 5). The genus *Heterosigma* contains a new species, *H. minor*, described in 2016 (Engesmo et al., 2016). These two species of *Heterosigma* are indistinguishable in the 18S rDNA V8–V9 region analyzed in this study. However, *H. minor*



**TABLE 1** List of HAB species and nori bleaching/fish kill species detected by metabarcoding in this study, their harmful effects, and their numbers of unique sequences and reads obtained in this study.

Genus	Species	Harmful effects and toxins of each HAB species	Unique sequence numbers	Numbers of reads
<i>Alexandrium</i>	<i>affine</i>	ichthyotoxicity (Nguyen-Ngoc Lim, 2004)	3	132
	<i>hiranoi/pseudogonyaulax</i>	goniodomin A from <i>A. hiranoi</i> (Murakami et al., 1988; 1998) goniodomin A from <i>A. pseudogonyaulax</i> (Triki et al., 2016)	4	552
	<i>leei</i>	ichthyotoxicity (Tang et al., 2007 and Shikata et al., 2020)	10	183
	<i>pacificum</i> (Group IV)	paralytic shellfish toxins (neosaxitoxin and gonyautoxins 1–6) (Wang et al., 2005; Li et al., 2011; Brown et al., 2021)	9	591
	<i>tamiyavanichii</i>	paralytic shellfish toxins (neosaxitoxin and gonyautoxins 1–5) (Hashimoto et al., 2002; Lim et al., 2006; Sagara et al., 2010)	2	177
<i>Azadinium</i>	<i>'poporum'</i> <sup>1</sup>	azaspiracids (Tillmann et al., 2011; 2018, Krock et al., 2015; Luo et al., 2017; 2018)	3	85
<i>Chattonella</i>	<i>marina</i>	ichthyotoxicity (Shikata et al., 2021)	2	827
<i>Fibrocapsa</i>	<i>japonica</i>	ichthyotoxicity (de Boer et al., 2009; 2012)	24	5,560
<i>Heterocapsa</i>	<i>'circularisquama'</i> <sup>2</sup>	bivalve mortality (Matsuyama, 2012)	3	228
<i>Heterosigma</i>	<i>'akashiwo'</i> <sup>3</sup>	ichthyotoxicity (Flores-Leñero et al., 2022 and Astuya et al., 2015; 2018; 2023)	2	364
<i>Skeletonema</i>	spp.	nori bleaching and fish kill (Hori et al., 2019; Imai et al., 2006; 2021, Kent et al., 1995; Minamiura and Yamaguchi, 2019; Sakamoto et al., 2021)	23	3,742

<sup>1</sup>The sequences of *Azadinium dalianense/poporum* (Supplementary Figure 4) were determined to be those of *A. 'poporum'* in this study, because *A. poporum* has been found at various locations in Japan (Takahashi et al., 2021b).

<sup>2</sup>The sequences of *Heterocapsa circularisquama* (Supplementary Figure 6) were determined to be those of *H. 'circularisquama'* in this study, because *H. circularisquama* has been found at Uranouchi Inlet (Shiraishi et al., 2008).

<sup>3</sup>The sequences of *Heterosigma akashiwo/minor* (Supplementary Figure 5) were determined to be those of *H. 'akashiwo'* in this study, because *H. akashiwo* has been found at various locations in Japan (Engesmo et al., 2016; Imai et al., 2021; Mehdizadeh Allaf, Malihe, 2023).

has only been described from a strain isolated from Virginia, USA (strain ARC HA0504-1), and no Japanese strain has been isolated to date (Engesmo et al., 2016; Imai et al., 2021; Mehdizadeh Allaf, 2023). Therefore, we determined the unique sequences in the phylogenetic tree as *H. 'akashiwo'* (Supplementary Figure 5).

Three unique sequences were found in the genus *Heterocapsa*, but the position of those sequences in the phylogenetic tree of the genus *Heterocapsa* was difficult to identify at the species level, since those sequences belonged to one clade along with several other species of the genus *Heterocapsa* (Supplementary Figure 6). This is because the 18S rDNA V8–V9 region, which is the target region for metabarcoding in this study, cannot identify each species of the genus *Heterocapsa*. However, considering that there are reports of bivalve mortality caused by *H. circularisquama* in Uranouchi Inlet (Horiguchi, 1995; Imai et al., 2006; Matsuyama, 2012; Sakamoto et al., 2021; Shiraishi et al., 2008), these three sequences were considered as *H. circularisquama* and named as *H. 'circularisquama'* (Table 1).

The blooms of *Skeletonema* have been reported to be responsible for the color bleaching of nori (*Pyropia* spp.) in Japan (Imai et al., 2006, Table 1) and gill lesions and mortality of Atlantic salmon *Salmo salar* in British Columbia (Kent et al., 1995), unique sequences assigned to this genus were treated as HAB species in this study (Supplementary Figure 7).

### 3.3 Monitoring data of HAB species and water quality survey

The HAB monitoring data provided by the Kochi Prefectural Fisheries Experiment Station for fishery damage in Uranouchi Inlet between 1984 and 2017 showed that the largest fishery damage on record occurred in 2001 in this inlet, with the damage amounting to 60 million yen (Table 2).

Water quality survey results from 1980 to 2020 showed that DIN-N, DON-N, DOP-P, T-N and T-P stayed high in 2005, while inorganic nitrogen (NH<sub>4</sub>-N, NO<sub>2</sub>-N, NO<sub>3</sub>-N and DIN-N) and inorganic phosphorus (PO<sub>4</sub>-P) were intermittently high from 1980 to 2020 (Table 3).

### 3.4 Vertical distribution of HAB species

The vertical distribution of HAB species analyzed via a heatmap revealed that the eleven HAB species could be divided into three groups (Figure 1). The first group was composed of six species found in samples from almost all the sediment layers: *A. hiranoi/pseudogonyaulax*, *C. marina* complex, *F. japonica*, *H. 'circularisquama'*, *H. 'akashiwo'* and *Skeletonema* spp.

TABLE 2 Summary of the dominant HAB species of the red tides and the economic damage to fish aquaculture in Uranouchi Inlet, compiled by the Kochi Prefectural Fisheries Experiment Station.

Year	Species observed during red tide out-break (concentration : cells/L)				Damaged fish species	Damage quantity	Damage amount (multiplied by thousands of JPY)
1984	<i>Heterosigma</i> sp. (ND)				Japanese amberjack	2,500 fishes	1,950
1991	<i>Chattonella marina</i> (640)				Japanese amberjack	21,500 fishes	8,920
1992	<i>Heterosigma akashiwo</i> (4,880)				greater amberjack	600 fishes	540
1993	<i>Chattonella marina</i> (10,533)	<i>Heterosigma akashiwo</i> (ND)	<i>Karenia mikimotoi</i> (ND)		Japanese amberjack, striped jack	Japanese amberjack 50,000 fishes, striped jack 20,000 fishes	35,000
1994	<i>Chattonella marina</i> (ND)	<i>Karenia mikimotoi</i> (35,000)			red sea bream	800 fishes	800
1994	<i>Chattonella marina</i> (3,300)				striped jack	1,000 fishes	1,500
1997	<i>Chattonella antiqua</i> (8,300)				Japanese amberjack	15,000 fishes	20,000
2001	<i>Heterosigma akashiwo</i> (113,800)				red sea bream, yellowtail	red sea bream 2,000,000 fishes, yellowtail 600,000 fishes	60,000
2001	<i>Fibrocapsa japonica</i> (1,600)	<i>Chattonella</i> spp. (ND)			greater amberjack	10,000 fishes	ND
2002	<i>Chattonella antiqua</i> (4,100)	<i>C. marina</i> (ND)			Japanese amberjack	1,100 fishes	270
2003	<i>Chattonella antiqua/marina</i> (25,700)				Japanese amberjack	54,000 fishes	26,000
2003	<i>Chattonella antiqua/marina</i> (4,134)				Japanese amberjack, greater amberjack	Japanese amberjack 6,180 fishes, greater amberjack 400 fishes	1,600
2004	<i>Chattonella marina</i> (ND)	<i>Karenia mikimotoi</i> (16,664)			greater amberjack, red sea bream, abalone	greater amberjack 343 fishes, red sea bream 269 fishes, 250,000 abalones	ND
2006	<i>Heterosigma akashiwo</i> (27,800)				red sea bream, striped jack	ND	ND
2006	<i>Chattonella marina</i> (ND)	<i>Karenia mikimotoi</i> (52,580)			greater amberjack, red sea bream	greater amberjack 2,000 fishes, red sea bream 300 fishes	ND
2007	<i>Chattonella marina</i> (15,400)				Japanese amberjack, greater amberjack	Japanese amberjack 40 fishes, greater amberjack 40 fishes	20
2008	<i>Chattonella</i> spp. (18,700)				Japanese amberjack, greater amberjack, red sea bream, striped jack	ND	5,856

(Continued)

TABLE 2 Continued

Year	Species observed during red tide out-break (concentration : cells/L)				Damaged fish species	Damage quantity	Damage amount (multiplied by thousands of JPY)
2009	<b><i>Chattonella</i> spp.</b> (13,320)				Japanese amberjack	ND	ND
2009	<b><i>Heterosigma akashiwo</i></b> (12,400)				striped jack	220 fishes	ND
2010	<b><i>Chattonella marina</i></b> (6,250)	<i>Karenia mikimotoi</i> (1,640)			yellowtail	1,500 fishes	ND
2011	<b><i>Chattonella</i> spp.</b> (16,500)				Japanese amberjack, greater amberjack, bluefin tuna	Japanese amberjack 2,200 kg, greater amberjack 6,960 kg, bluefin tuna 4.2 kg	8,371
2011	<b><i>Chattonella</i> spp.</b> (3,080)	<i>Karenia mikimotoi</i> (1,170)			Japanese amberjack	20,200 kg	14,650
2012	<b><i>Chattonella</i> spp.</b> (5,230)	<b><i>Fibrocapsa japonica</i></b> (1,080)	<i>Karenia mikimotoi</i> (8,875)	<i>Dictyocha fibula</i> (4,690)	yellowtail, greater amberjack, red sea bream	yellowtail 50 fishes, greater amberjack 504 fishes, red sea bream 773 fishes	ND
2012	<b><i>Chattonella</i> spp.</b> (5,230)	<b><i>Fibrocapsa japonica</i></b> (1,080)	<i>Karenia mikimotoi</i> (27,300)	<i>Dictyocha fibula</i> (4,690)			
2013	<b><i>Heterosigma akashiwo</i></b> (515,000)				greater amberjack	5 fishes	ND
2014	<b><i>Chattonella marina</i></b> (6,800)				greater amberjack, red sea bream	greater amberjack 10,000 fishes, red sea bream 172 fishes	ND
2014	<b><i>Chattonella marina</i></b> (6,800)						
2015	<b><i>Chattonella</i> spp.</b> (61)				Japanese amberjack, greater amberjack, red sea bream	Japanese amberjack 2,900 fishes, greater amberjack 6,990 fishes, red sea bream 18,400 fishes	23,890
2015	<b><i>Chattonella</i> spp.</b> (170,000)	<i>Karenia mikimotoi</i> (ND)					
2015	<b><i>Chattonella</i> spp.</b> (11,300)	<i>Karenia mikimotoi</i> (ND)					
2015	<b><i>Chattonella</i> spp.</b> (4,900)	<i>Karenia mikimotoi</i> (ND)					
2016	<i>Pseudochattonella verruculosa</i> (450)				greater amberjack	110 fishes	ND
2017	<b><i>Heterosigma akashiwo</i></b> (20,500)				red sea bream	2,900 fishes	2,540
2017	<b><i>Chattonella</i> spp.</b> (6,650)				yellowtail, greater amberjack, bluefin tuna	yellowtail 3,500 fishes, greater, amberjack 500 fishes, bluefin tuna 60 fishes	6,330

ND, No data.

HAB species highlighted by bold: HAB species detected in the metabarcoding that were included in this study.

HAB species shown by fine: HAB species detected by metabarcoding but excluded from analysis in this study.

The second group of *A. affine*, *A. pacificum* (Group IV), and *A. tamiyavanichii* was not detected or was detected at low abundance in the upper layers of the core sample (Figure 1, URA01–08), whereas these species were detected in the deeper layers (under URA09 and URA10, whose years of occurrence were estimated to be 1980s).

The third group was composed of two HAB species, *A. leei* and *A. poporum*, which were not detected in the deep layers, unlike the URA10 and URA11 samples, respectively, but were detected in the upper layers (Figure 1, URA01–URA11).

## 4 Discussion

### 4.1 DNA of HABs found by metabarcoding

Among the detected HAB species, *Alexandrium hiranoi* is known to form resting cysts in the winter and can remain dormant until the environmental conditions become suitable for germination (Kita et al., 1985). *A. leei* (Anderson et al., 2012; Shikata et al., 2020), *A. pseudogonyaulax* (Anderson et al., 2012; Lassus et al., 2016; Montresor, 1995; Montresor and Marino, 1996), *A. affine*, *A. pacificum* (Group IV) and *A. tamiyavanichii* are also known to form cysts (Anderson et al., 2012; Nguyen-Ngoc, 2004). The following other HAB species are also known to form resting cysts in the sediment: *A. poporum* (Gu et al., 2013a; Luo et al., 2018), *C. marina* complex (Imai and Yamaguchi, 2012; Ishikawa et al., 2022; Katano et al., 2014), *F. japonica* (Cucchiari et al., 2010; Yoshimatsu, 1987), *H. circularisquama* (Shiraishi et al., 2008), *H. akashiwo* (Mehdizadeh Allaf, 2023; Mehdizadeh Allaf and Trick, 2023) and *Skeletonema* spp (Ellegaard and Ribeiro, 2018; McQuoid and Hobson, 1996; Stenow et al., 2020). Therefore, those DNAs detected in the sediment samples have the possibility of originating from cysts or from DNA adsorbed on humic acids (Lewis et al.,

2023; Pedersen et al., 2015; Saeki et al., 2011) in the sediment samples.

### 4.2 Insights into the occurrence trends of HAB species group

From the vertical distribution of eighteen HAB species analyzed by heatmap, the first six HAB species group (*A. hiranoi/pseudogonyaulax*, *C. marina* complex, *F. japonica*, *H. circularisquama*, *H. akashiwo* and *Skeletonema* spp.) which appeared in almost every sediment sample suggest that these six cyst-forming species occurred continuously throughout the ages, and DNA derived from the cysts or cells were deposited in the bottom sediment resulting in their detection in all sediment samples. Since 1984, when records began to be kept by the Kochi Prefectural Fisheries Experimental Station, four genera, *Chattonella*, *Fibrocapsa*, *Heterocapsa*, and *Heterosigma*, have repeatedly formed red tides and caused economic damage to aquaculture (~60 million JPY, Table 2). Although there are no records prior to 1984, these records after 1984 seem to correspond to the existence of those four genera in the sediments during that period (Figure 1, URA01–URA09).

According to Kochi Prefectural Fisheries Experimental Station, four *Alexandrium* species (*A. affine*, *A. pacificum* (Group IV), *A. tamiyavanichii* and *A. leei*) detected by metabarcoding have not been reported by direct cell counting, but three Raphidophyta (genera *Chattonella*, *Fibrocapsa*, and *Heterosigma*) have been reported (Table 2). The reason for this difference is the number of copies of 18S rDNA per cell in *Alexandrium* spp. and Raphidophyta. It is known that the copy number of 18S rDNA per cell in the genus *Alexandrium* is higher than that in Raphidophyta (Yarimizu et al., 2021). Therefore, it is likely that metabarcoding would have detected Raphidophyta but

TABLE 3 Summary of water quality survey results in Mitsumatsu station near Menokuso Station in Uranouchi Inlet, where the core sample used in this study was collected, conducted by Kochi Prefectural Fisheries Experiment Station and Fisheries Research Institute, Japan Fisheries Research and Education Agency.

Year	NH <sub>4</sub> -N (mg/L)	NO <sub>2</sub> -N (mg/L)	NO <sub>3</sub> -N (mg/L)	PO <sub>4</sub> -P (mg/L)	DIN- N (mg/L)	DON- N (mg/L)	DOP- P (mg/L)	T-N (mg/L)	T-P (mg/L)
1980	0.080	0.009	0.034	0.028	0.123	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>
1985	0.119	0.004	0.008	0.057	0.131	0.128	0.014	0.260	0.070
1987	0.086	0.142	0.043	0.077	0.271	0.068	0.046	0.339	0.123
1990	0.184	0.018	0.009	0.082	0.211	0.092	0.004	0.303	0.086
1995	0.196	0.003	0.005	0.074	0.204	0.122	0.012	0.326	0.086
2000	0.013	0.121	0.041	0.067	0.175	0.102	0.011	0.277	0.078
2005	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	0.140	0.365	0.840	0.119	1.205	0.259
2010	0.073	0.005	0.005	0.018	0.084	0.189	0.011	0.272	0.029
2015	0.158	0.010	0.007	0.030	0.175	0.063	0.009	0.238	0.038
2020	0.003	0.191	0.053	0.062	0.247	0.067	0.008	0.314	0.070

<sup>a</sup>Data not available.

Data from 2007 onward are available to the public online page in Kochi Prefectural Fisheries Experiment Station website written in Japanese (<https://www.pref.kochi.lg.jp/soshiki/040409/akashiojoho.html>).



*Alexandrium* was not. This is a weakness of the occurrence analysis by metabarcoding and should be analyzed by quantitative metabarcoding in the future (Ushio et al., 2018; Sato et al., 2021; Tsuji et al., 2022).

The reason why *A. 'poporum'* was detected by metabarcoding but its occurrence has not been recorded may be because the size of this species is small and difficult to identify under microscopic observation (Takahashi et al., 2021b; Tillmann et al., 2011). Considering these issues, analysis of HAB species in bottom sediments by metabarcoding is useful for clarifying the occurrence history of HAB species.

In relation with the second HAB species group (*A. affine*, *A. pacificum* (Group IV), and *A. tamiyavanichii*) in the heatmap, *Alexandrium* spp. blooms occur on a large scale in Osaka Bay and Hiroshima Bay in the Seto Inland Sea, Japan, when nutrient concentrations such as DIN and PO<sub>4</sub> are less than 12.8 µM and 0.4 µM, respectively (Itakura et al., 2002; Yamamoto, 2019). Considering these issues, it has been reported that conditions suitable for *Alexandrium* spp. proliferation may involve oligotrophic or mesotrophic waters rather than eutrophic waters (Itakura et al., 2002; Natsuike et al., 2018a, 2018b; Yamamoto, 2019). Therefore, the bloom formation of the *Alexandrium* species in Uranouchi Inlet may have been suppressed by eutrophication caused by aquaculture after 1977–1988 (Table 3), as discussed later.

There are two possibilities for why the third HAB species group (*A. leei* and *A. 'poporum'*) in the heatmap were not detected in deeper samples than the URA11 and URA12 samples, whose years estimated by radiometric dating (Takahashi et al., 2021a) were 1977 and 1954. First, these two species did not occur in Uranouchi Inlet prior to 1977 or 1954 and appeared after those years; second, the cysts that formed prior to 1977 or 1954 might have decomposed since the cysts of these two HAB species are not highly durable. Regarding the first possibility, yellowtail aquaculture started in Uranouchi Inlet in 1959, and the aquaculture industry may have caused environmental changes such as eutrophication, as discussed later in the enclosed inlet, which may have enhanced bloom formation in these two species. The cyst durability of these two species should be investigated to clarify which possibility is conceivable.

### 4.3 Replacement of HAB species groups in the sediment of Uranouchi Inlet

Possible causes of the change in HAB species composition after 1977–1988 (URA09–11) include the following two possibilities: first, eutrophication of Uranouchi Inlet due to the start of aquaculture, and second, climate change, such as global warming. Regarding the first possibility, the total N (T-N) and total P (T-P) concentrations in Uranouchi Inlet in 1985 were 0.260 mg/L and 0.070 mg/L, respectively (Table 3). These values suggest that the seawaters in Uranouchi Inlet were eutrophicated or polluted at that time, based on the criterion of eutrophication (T-N: 0.220–0.650 mg/L, T-P: 0.03–0.09 mg/L, Tavakoly Sany et al., 2014) and

pollution in coastal waters ('low-level' T-N pollution: 0.252–0.308 mg/L; and 'very high level' T-P pollution: > 0.031 mg/L, Smith, 2003). It is possible that the eutrophication in Uranouchi Inlet may have occurred as a result of the proliferation of aquaculture in Uranouchi Inlet from the 1960s to the 1970s, which resulted in changes in HAB species composition from 1977–1988. However, information on nutrient concentrations in the years prior to the 1980s is not available. This hypothesis is supported by the finding that *Skeletonema* spp., a known indicator of eutrophication in the Seto Inland Sea of Japan (Itakura and Yamaguchi, 2007; Nishikawa et al., 2010; Yamada et al., 1980a, 1980b, 2011), have been detected in greater numbers in Uranouchi Inlet since 1977–1988 (Figure 1). On the other hand, there is a gap of more than a decade between the start of aquaculture in Uranouchi Inlet (1959) and the transition timing of the HAB species groups occurred (1977–1988). This gap may be due to eutrophication caused by nutrients increasing beyond the environmental capacity while the aquaculture was gradually expanded since the start of aquaculture was not sudden on a large scale.

Second, the sea surface temperature (SST) around Japan has increased due to global warming, and the annual average SST in the northwestern Pacific around Japan has shown an increasing trend (1.24 °C/100 years, Japan Meteorological Agency, 2023). The annual average SST in Uranouchi Inlet increased by 0.19 °C/decade from the 1970s to the 2010s (Tanaka et al., 2012). Such SST trends in Uranouchi Inlet and nearby sea areas may increase blooms of HAB species such as *A. leei*, which have been reported to occur in tropical and subtropical areas, such as Malaysia (Usup et al., 2002), Singapore (Tang et al., 2007), Thailand (Kodama et al., 1982), Vietnam (Nguyen-Ngoc, 2004), and China (Gu et al., 2013b). This hypothesis was supported by the report that *A. leei* recently formed blooms and caused fish mortality in Nomi Bay in 2017 (Shikata et al., 2020), which is close to Uranouchi Inlet. Although it is difficult to directly link such changes in the bloom formation of HAB species with global warming, it may be important to consider global warming as at least one of the factors that caused the changes in the occurrence trends of HAB species in Uranouchi Inlet observed in this study.

In this study, we revealed the presence of eleven HAB species, with notable shifts from approximately 1977–1988 in Uranouchi Inlet, Kochi, Japan. This shift corresponded to two hypotheses: the development of aquaculture and the resulting eutrophication, or sea surface temperature rising due to global warming. Moreover, metabarcoding using vertical core sediment samples provides footprints of how HAB species composition has changed and maybe be affected by anthropogenic environmental changes.

### Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

## Author contributions

HF: Conceptualization, Writing – original draft, Methodology, Visualization, Data curation, Formal analysis, Investigation. CG: Methodology, Data curation, Writing – original draft. TN: Data curation, Writing – original draft. KT: Writing – original draft, Resources. KK: Formal analysis, Writing – original draft, Data curation. TK: Data curation, Writing – original draft, Formal analysis. KN: Writing – original draft, Funding acquisition. MA: Writing – review & editing, Writing – original draft, Conceptualization, Supervision, Funding acquisition, Visualization, Validation.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/frpro.2025.1612811/full#supplementary-material>

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