Monitoring of the toxic dinoflagellate *Alexandrium catenella* in Osaka Bay, Japan using a massively parallel sequencing (MPS)-based technique

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ABSTRACT

Since 2002, blooms of *Alexandrium catenella* sensu Fraga et al. (2015) and paralytic shellfish toxicity events have occurred almost yearly in Osaka Bay, Japan. To better understand the triggers for reoccurring *A. catenella* blooms in Osaka Bay, phytoplankton community was monitored during the spring seasons of 2012–2015. Monitoring was performed using massively parallel sequencing (MPS)-based technique on amplicon sequences of the 18S rRNA gene. Dense blooms of *A. catenella* occurred every year except in 2012, however, there was no significant correlation with the environmental parameters investigated. Plankton community diversity decreased before and middle of the *A. catenella* blooms, suggesting that the decline in diversity could be an indicator for the bloom occurrence. The yearly abundance pattern of *A. catenella* cells obtained by morphology-based counting coincided with the relative sequence abundances, which supports the effectiveness of MPS-based phytoplankton monitoring.

1. Introduction

Planktonic microbes, including protozoa, algae, fungi, and small metazoans, are ubiquitous and diverse in marine ecosystems (Jobard et al., 2010; Caron et al., 2012; de Vargas et al., 2015; Yu et al., 2015; Hu et al., 2016; Hirai et al., 2017). These microeukaryotic organisms are important components of marine food webs as predators, producers, decomposers, and parasites, and thus, play key roles in the functioning of marine ecosystems (Azam et al., 1983; Sherr and Sherr, 1994; Duffy and Stachowicz, 2006; Sherr et al., 2011). Minor variations in the structure and composition of these communities may cause changes at different trophic levels (Gamfeldt et al., 2005; Anjusha et al., 2013; Plum et al., 2015; Camarena-Gómez et al., 2018). As most microeukaryotic plankton assemblages are highly susceptible to environmental deterioration and pollution (Smayda, 1998; Stomp et al., 2008), it is necessary to investigate and monitor temporal variations in the biodiversity within their communities to characterize the health of marine ecosystems (Ptacnik et al., 2008; Beaugrand et al., 2010; Johnson et al., 2011; Spilling et al., 2018). Another important aspect of

monitoring biodiversity is the detection of harmful algal bloom (HAB) species as their blooms can have a negative influence on several sectors, e.g. aquaculture, tourism, public health, damage to the marine ecosystems (Zingone and Enevoldsen, 2000; Anderson, 2009; Dyson and Huppert, 2010; Berdalet et al., 2016).

Studies on microbial community diversity have traditionally relied on morphology-based identification and quantification done by microscopic observation (e.g. Taylor and Waters, 1982; Balech, 1995; Tomas, 1997; Edler and Elbrächter, 2010; Nishikawa et al., 2010; Wollschläger et al., 2015). Reliable identification of these microorganisms by light microscopy (LM) may be challenging due to small size as well as lack of distinctive and taxonomical rigorosity in morphological characteristics (e.g. Rhodes, 1998; Nübel et al., 1999; John et al., 2005; Aguilera et al., 2006; Galluzzi et al., 2008). Massively parallel sequencing (MPS) is a powerful method enabling detection of species unidentifiable by LM (e.g. Sogin et al., 2006; Caron et al., 2012; Nagai et al., 2017; Gran-Stadniczeňko et al., 2018) and revealing the hidden diversity of microbial communities in environmental samples (Tanabe et al., 2016; Nagai et al., 2016a,b; Dzhenbekova et al., 2017;

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Fig. 1. Sampling stations (Stations 12 and 19) in Osaka Bay, Japan.

Sze et al., 2018).

Effectiveness of MPS to detect many species simultaneously is especially relevant to reveal the presence of HAB species/genera (e.g. Dzhenbekova et al., 2017; Elferink et al., 2017; Moreno-Pino et al., 2018; Nagai, 2018) and gather information on their appearances as some species may be present sporadically (Nagai et al., 2017) or during specific seasons (Sildever et al., 2019). This information is prerequisite for monitoring by other molecular methods, e.g. qPCR and microarrays, which are limited by the number of species that can be detected at the same time (Medlin and Orozco, 2017) and by the need for specific primers to detect species of interest from the environmental samples (Garneau et al., 2011; Nagai et al., 2016c; Penna et al., 2013; Ruvindey et al., 2018).

The microalgal genus *Alexandrium* Halim, 1960 (Dinophyceae, Gonyaulacales) currently contains 34 species (Balech, 1995; MacKenzie and Todd, 2002; Montessoro et al., 2004; Guiry and Guiry, 2019), including 12 that are known to produce paralytic shellfish toxins (PSTs) (Anderson et al., 2012; Moestrup et al., 2019), responsible shellfish toxicity and the resulting paralytic shellfish poisoning (PSP) throughout the world (Anderson et al., 2012). To provide an early warning for the fisheries industry and avoid adverse effects on human health, monitoring the temporal variation in the abundance of *Alexandrium* spp. has become indispensable (Itakura et al., 2002; Weise et al., 2002; Anderson et al., 2005; Shimada et al., 2012; Laanaia et al., 2013; Yamamoto et al., 2013; 2017). In this study, *A. catenella* sensu Fraga et al. (2015) was monitored in relation to plankton biodiversity and environmental parameters as this toxic dinoflagellate is present in all Japanese coastal waters (Fukuyo, 1985; Sekiguchi et al., 1986; Uchida et al., 1980; Yamamoto et al., 2009) and has become a conspicuous species in southwestern Japan since 1990s (Yamamoto and Yamasaki, 1996; Kotani et al., 1998).

In Osaka Bay, PSTs were first reported in bivalves in the spring of 2002 (Hamano et al., 2002; Yamamoto, 2004). Since then, the growing number of *A. catenella* blooms and PST contamination of bivalves have been observed almost yearly (Yamamoto et al., 2017; Yamamoto and Oikawa, 2017). The appearance of *A. catenella* blooms in southwestern Japan may be related to changes in phytoplankton species diversity. From the late 1970s to early 1980s diatoms were the most dominant group causing HABs in the Seto Inland Sea, including Osaka Bay, whereas raphidophytes became significant in the mid-1980s and were replaced by dinoflagellates in 1990s (Yamamoto, 2003). The changes in dominating HAB species have been explained by the reduction of DIN and DIP concentrations since the 1980s (Yamamoto, 2003; Abo and Yamamoto, 2019). On a shorter perspective, the development of *A. catenella* blooms are also influenced by other factors, as the species has

been detected yearly from 2002 to 2016 in Osaka Bay, however, the blooms did not occur every year (Yamamoto et al., 2017). In addition to influence of physical factors, e.g. water column stability (Yamamoto et al., 2002; 2013), reduction in DIP concentrations (Yamamoto et al., 2017) and exhaustion of nutrients by large diatoms resulting in reduced spring bloom intensity (Itakura et al., 2002) have been suggested to contribute to the development of *A. catenella* blooms. This suggests that changes in biodiversity may allow predicting the occurrence of *A. catenella* blooms.

Thus, the aim of this study was to investigate the temporal variation in the diversity of microeukaryotic plankton assemblages to reveal patterns in *A. catenella* and other phytoplankton species appearance to better understand the triggers for reoccurring *A. catenella* blooms in Osaka Bay. Weekly sampling to monitor *A. catenella* and other microeukaryotic species in Osaka Bay was conducted from February to May 2012–2015 and analyzed these samples by using an MPS-based technique.

2. Materials and methods

2.1. Sampling and DNA extraction

Seawater samples were obtained weekly using a 10-m hose (diameter, 25 mm) from February to May during 2012–2015 ($n = 122$) at two sampling stations (Stations 12 and 19) in Osaka Bay (St. 12, $34^{\circ} 30.36' N$, $135^{\circ} 16.83' E$; St. 19, $34^{\circ} 28.20' N$, $135^{\circ} 19.83' E$), Japan (Fig. 1). The sea surface temperature was measured with a thermometer on-site and the salinity was measured using a salinometer (Digi-auto Model 3-G, Tsurumi Seiki Co. Ltd.) in the laboratory. Water samples for nutrient analysis were collected biweekly and filtered through a GF/C filter. Dissolved inorganic nitrogen ($DIN = NO_3 + NO_2 + NH_4$), phosphate (PO_4), and silicic acid ($Si(OH)_4$) were analyzed using an autoanalyzer (swAAt, BLTEC). Monthly precipitation, number of rainy days, maximum wind speed, and peak gusts in the sampling area (Kankujima station) during February–April of 2012–2015 were downloaded from the official website of the Japan Meteorological Agency (<http://www.jma.go.jp/>; accessed 10.04.2019).

All the plankton in the samples were trapped by filtering 500 mL of seawater through 8- μm pore-size polycarbonate filters (Nuclepore membrane; GE Healthcare, Tokyo, Japan), followed by their filtration through 1- μm pore-size filters (GE Healthcare). The filters were stored in a deep freezer ($-80^{\circ} C$) until use. For DNA extraction, a 5% Chelex® suspension (Chelex 100 Molecular Biology Grade Resin; Bio-Rad Laboratories Inc., Richmond, CA) was prepared by dispersing the resin in ultra-pure water (Nagai et al., 2012). For effective DNA extraction

from plankton components trapped on the filters, the filters were cut in half, placed in 1.5-mL tubes (A.150; Assist; Tokyo, Japan), and 150 μ L of 5% Chelex buffer was added. The plankton cells were crushed using a pellet pestle cordless motor (KontesGlass; Vineland, NJ, USA) for 60 s; 350 μ L of buffer was then added to make up a final volume of 500 μ L. Following this, DNA was extracted by heating the contents of the 1.5-mL tubes at 97 °C for 20 min (Tanabe et al., 2016). The DNA extracted from the 8- μ m and 1- μ m filters was mixed equally (50 μ L + 50 μ L) and used as template DNA.

2.2. Enumeration of *Alexandrium catenella* by microscopic observation

The seawater samples were also used to enumerate the abundance of the PSP-causing dinoflagellate *A. catenella* as well to compare the relative abundance of *A. catenella* to the relative number of sequences per sample. One liter of seawater was sieved through a 10- μ m nylon mesh and the plankton components were concentrated until they reached a volume of 10 mL. The abundance of *A. catenella* in 1 mL of the concentrated seawater was counted by morphology-based observation using an inverted microscope (TE-300, Nikon, Tokyo, Japan).

2.3. Polymerase chain reaction amplification and 454 pyrosequencing

A set of universal primer pairs for amplification of the 18S rRNA gene was used to amplify the V7–9 hypervariable regions (SSR-F1, 289, F: TGGAGYGATTGTCTGGTTDATTCCG; SSR-R1, 772, R: TCACCTACGG AACCTTGTTACG) (Tanabe et al., 2016); six-base pair (bp) tags were added to the 5'-end of the primers for recognition of each sample in metagenetic analysis. The amplification reaction was performed using a thermal cycler (PC-808; ASTEC; Fukuoka, Japan) in a reaction mixture (50 μ L) containing 2.0 μ L template DNA (< 1 ng); 0.2 mM each of the dNTPs; 1 $\times$ PCR buffer; 1.5 mM Mg²⁺; 1.0 U KOD-Plus-ver.2 (TOYOBO; Osaka, Japan), which has intensive 3'→5' exonuclease activity; and 1.0 μ M each of the primers. The PCR cycling conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 25 cycles with each having periods of 94 °C for 15 s, 56 °C for 30 s, and 68 °C for 1 min. PCR amplification was verified by agarose gel electrophoresis. The PCR products were purified using a High-Pure PCR Product Purification Kit (Roche; Basel, Switzerland) and eluted in 35 μ L of elution buffer following the manufacturer's protocol. Amplified PCR products were quantified using a Qubit 2.0 Fluorometer (Life Technology, Carlsbad, CA, USA) and mixed equally. Amplicon sequencing was performed using the Roche 454 GS-XLR70 Titanium sequencing platform. A 454-pyrosequencing library was constructed using the GS Titanium Rapid Library Preparation Kit (Roche Diagnostics; Branford, CT, USA); pyrosequencing was then performed using a Roche 454 GS FLX + sequencer according to the manufacturer's protocol (Roche Diagnostics; Branford, CT, USA).

2.4. Treatment processes for the MPS data and operational taxonomic unit pick up

Nucleotide sequences were demultiplexed based on 5' MID-tag and primer sequences (MID = multiplex identifier). As the sequencing adapters were ligated after the PCR, each sample sequence contained sequences for both strands. Both strands shared the same MID-tag sequence, but the 5' primers were distinct, therefore, different strand sequences were also sorted. The sequences containing (a) > 1 bp mismatch in the MID-tag, (b) > 2 bp mismatches in the primer sequences, (c) N in both the MID-tag and primer sequences, and (d) monopolymers longer than 9 bp were trimmed from the sequences at both ends. The 3'-tails with an average quality score of less than 25 at the end of the last 50-bp window were also trimmed from each sequence. Sequences longer than 430 bp were truncated to 430 bp by trimming their 3'-tails. Trimmed sequences shorter than 400 bp were filtered out. Subsequently, each sequence was aligned with template sequences

using multiple alignments as described previously (SILVA release 111, <http://www.arb-silva.de/>), and only the sequences that were aligned in appropriate positions were extracted. The demultiplexing, trimming, filtering, and multiple alignment processes were performed using the screen.seqs, trim.seqs, filter.seqs, and align.seqs, commands, respectively, in Mothur based on the SOP (http://www.mothur.org/wiki/454_SOP). The reverse strand sequences were converted to their reverse-complement sequences. Erroneous and chimeric sequences were detected and removed using pre.cluster (diffs = 4) and chimera.uchime (minh = 0.1; http://drive5.com/usearch/manual/uchime_algo.html) commands in Mothur, respectively. In addition, singletons were removed. The forward strand sequences and reverse-complement sequences of the reverse strand for each variable region were separately processed. The remaining sequences were assembled using the clmergclass command in Claident (Tanabe, 2012a) and Assams-assembler v0.1.2013.07.19 (Tanabe, 2012b) with sequence identity thresholds of 0.99 (0.01 distance radius in sequence space) and a minimum overlap length of 100 bp. Sequence assembly of homologous amplicon sequences by software, Claident, and Assams, is equivalent to complete linkage clustering, except that the resulting sequences are not representative sequences but are only consensus sequences. The contig sequences were counted as operational taxonomic units (OTUs) and used for the taxonomic identification analysis given in the next subsection. Demultiplexed, filtered, but untrimmed sequence data were deposited in the DDBJ Sequence Read Archive under accession no. PRJDB4580.

2.5. Taxonomic identification of the OTUs

The selected OTUs were taxonomically identified as follows: for the BLAST search, a subset of NCBI non-redundant nucleotides (NT) consisting of sequences that satisfied the below-mentioned conditions was prepared. The sequences used to identify OTUs were downloaded from the GenBank based on the following search criteria: “ribosomal”, “rRNA” or “rdna”, and the search excluded keywords associated with “metagenome”, “uncultured” and “environmental”. The sequences of retrieved GenBank IDs were downloaded from the nucleotide database at the NCBI FTP server on March 11, 2019, and were used to construct a template sequence database. Subsequently, the taxonomic identification for each OTU was performed using a BLAST search (Cheung et al., 2010) in NCBI BLAST + 2.2.26 + (Camacho et al., 2009) with default parameters. The nucleotide subset described above was treated as a database, and all OTU representative sequences were treated as queries. Subsequently, taxonomic information was obtained from the BLAST top bit-score hit for each query sequence. Sequences were clustered to OTUs at $\geq 99.1\%$ similarity level. The raw OTU reads were normalized by rarefying to 1155 sequence reads to facilitate comparing biodiversity among samples (Weiss et al., 2017).

The relative abundance of OTUs among samples at the supergroup level was compared by counting the number of sequences at each level for the 122 samples. In this study, the supergroups were defined as Alveolata, Amoebozoa, Apusozoa, Archaeplastida, Excavata, Hacrobia, Metazoa, Opisthokonta, Rhizaria, Stramenopila, and Viridiplantae as done by Adl et al. (2005); Burki et al. (2007); Frommolt et al. (2008); Archibald (2009); Hampl et al. (2009); Okamoto et al. (2009), and Sakaguchi et al. (2009).

Presence of harmful algal bloom (HAB) species among the detected OTUs (non-rarefied dataset) was investigated by comparing the list of OTUs associated by a single species against the IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae (Moestrup et al., 2019). The non-rarefied dataset was used to detect the presence of all the toxic HAB species as rarefying may omit data on the presence of some OTUs (Weiss et al., 2017). Only OTUs with the tophit similarity > 0.99 were included. For *A. catenella*, the nomenclature suggested by Fraga et al. (2015) was followed.

2.6. Community diversity analysis

The numbers of shared OTUs among different sampling years (rarefied dataset) were counted and depicted as a Venn diagram by using the “vegan” package (v2.3-1, Oksanen et al., 2019) in R (R Core Team, 2019). Species richness was calculated as the number of OTUs representing community diversity among the assemblages. The Simpson's diversity index was calculated as the proportion of species i relative to the total number of species (P_i) multiplied by the natural logarithm of this proportion ($\ln P_i$). Simpson's diversity index measures the degree of concentration, when individuals are classified into types, and represents species evenness within the assemblage (Simpson, 1949). The index is calculated as the reciprocal of the sum of the squared P_i for all the species. The biodiversity of eukaryotic communities in the samples was evaluated by applying the Bray-Curtis similarity index and visualized by non-metric multidimensional scaling (NMDS) in “vegan”. The goodness of fit of the resulting NMDS mapping was assessed by a Shepard diagram (Fig. S1). Biodiversity data from different years and months were separately visualized by NMDS to investigate differences in biodiversity on different temporal scales. Influence of environmental parameters to the variability in biodiversity was investigated by fitting data on water temperature, salinity, DIN, PO_4 , and $\text{Si}(\text{OH})_4$ concentrations in the NMDS plot by envfit function. Differences in biodiversity between the years and months were investigated by PERMANOVA using the Bray-Curtis similarity index and 999 permutations and the assumption for homogeneity of multivariate dispersions was tested by ANOVA (betadisper function in “vegan”).

Pearson correlations were calculated between the environmental parameters, microscopic observations, relative abundances of *A. catenella* OTUs detected, number of different OTUs detected and Simpson's index for data from both stations by using the package “psych” (Revelle, 2019) in R. Since nutrient data was collected biweekly, the correlation analysis for both stations was performed using two datasets: $n = 26$ (all parameters) and $n = 61$ (nutrient data not included), P value was adjusted for multiple tests by using Bonferroni correction.

To investigate changes in species composition between the years with *A. catenella* blooms (2013–2015) and no bloom (2012), differences in average relative abundances were calculated for OTUs associated with microalgae (Station 12: $n = 270$, station 19: $n = 273$, Table S1). Changes in 30 OTUs showing the highest differences in abundances were investigated.

3. Results

3.1. Environmental conditions during the survey periods in Osaka Bay

Temporal variation in environmental conditions, such as monthly precipitation, number of rainy days, and maximum wind speed in the sampling area of Osaka Bay in February–April during 2012–2015 are shown in Table 1. In 2012, the average monthly precipitation and number of rainy days for February–April were greater than in 2013, 2014 and 2015, as well as than the 10-years average (2005–2014). Maximum wind speed and peak gusts were observed in April 2012, suggesting potentially greater vertical mixing of seawater in 2012 than in 2013, 2014, and 2015. Solar radiation time in 2013 was highest and followed by 2014, 2012, and 2015. In this study, water temperatures ranged from 7.5° C to 21.7° C at the two sampling locations during the survey period and increased steadily from February to April in each year and did not differ greatly among different years (Fig. 2). Salinity ranged from 26.0 to 32.4 during the survey period and decreased several times due to precipitation except for 2013 (Fig. 2). DIN, PO_4 and $\text{Si}(\text{OH})_4$ concentrations during the survey period were 0.1–25.6 μM , 0.01–0.73 μM , 0.1–23.5 μM , respectively. Concentrations of the inorganic nutrients were lower in 2013 and 2014 than in 2012 and 2015 (Fig. 3).

3.2. Detected community diversity

The number of sequences after the removal of sequence errors and chimeras varied from 1155 to 62,783 ($19,042 \pm 12,208$, mean $\pm$ SD) (data not shown). In the frequency distribution of the top BLAST hit similarity, 34.32% of the data scored 1.0 for the highest similarity with the reference sequences, and 63.57% scored > 0.98 . In total, 571 OTUs in plankton assemblages from 122 samples collected at two localities were detected (Table S2). Out of all the identified OTUs in the non-rarefied dataset, 13 were associated with the toxic HAB species (Moestrup, et al. 2019), including four *Alexandrium* species: *A. andersonii*, *A. catenella*, *A. minutum*, and *A. pacificum* (Table S2). In addition, two non-toxic *Alexandrium* species (*A. insuetum* and *A. leei*) were also detected. The detection limit of MPS-based monitoring of *A. catenella* was 1 pg, which is equivalent to 50 copies of rRNA genes (Nagai et al., 2016c), indicating that the technique is sufficiently sensitive for monitoring *A. catenella*.

The rarefied dataset contained 494 OTUs (Table S3), of which 209 OTUs (42.31%) were shared among four years, however, 21 (4.25%), 14 (2.83%), 41 (8.29%) and 27 OTUs (5.46%) were detected only in 2012, 2013, 2014, and 2015, respectively (Fig. 6). From all the OTUs detected (571, based on the non-rarefied dataset) 316 (55.34%) were identified as a single species, e.g. 69 as dinoflagellates, 30 as diatoms, 68 as zooplankton, 38 as ciliates (Table S2). Species richness with rarefaction was 62–119 OTUs (92 ± 16), 31–127 (79 ± 28), 16–105 (54 ± 21), 21–108 (57 ± 23), in 2012, 2013, 2014, and 2015, respectively (Fig. 4). Simpson's index ranged from 0.48 to 0.95 (0.82 ± 0.10), 0.23 to 0.96 (0.74 ± 0.21), 0.17 to 0.94 (0.64 ± 0.20) and, 0.14 to 0.94 (0.61 ± 0.20) in 2012, 2013, 2014, and 2015, respectively (Fig. 4). Samples in which the index was smaller than 0.5 was 3.12% (1 out of 32 samples), 18.17% (6/32), 21.87% (7/32), and 37.5% (12/32) in 2012, 2013, 2014, and 2015, respectively.

The NMDS analysis based on Bray-Curtis index ($k = 3$, stress 0.17), revealed that samples collected in 2012 clustered closer together in comparison to those collected in 2013, 2014, and 2015 (Fig. 5A). Conversely, the dispersed distribution of the samples collected in 2013, 2014, and 2015 suggested larger differences in the eukaryotic biodiversity among the samples, which is also visible from the Simpson's diversity index (Fig. 4). DIN ($P = 0.020$), PO_4 ($P = 0.009$), $\text{Si}(\text{OH})_4$ ($P = 0.029$), and water temperature ($P = 0.001$) contributed significantly to the variability in biodiversity, whereas the influence of salinity was not significant ($P = 0.051$) (Fig. 5A, B). The biodiversity in sampled communities was significantly different between months and years ($P < 0.001$), however, the homogeneity of multivariate dispersions was only met for dataset based on months ($P = 0.19$), but not for the dataset based on years ($P = 0.03$). Thus, it is not certain if the significant difference in biodiversity between the years reflects true dissimilarity between the years or differences in biodiversity within the years compared (Anderson et al., 2006).

3.3. Detection of the toxic dinoflagellate *Alexandrium catenella*

The abundance of *A. catenella*, the main causative species behind PSP in Osaka Bay, was enumerated using morphology-based observations from February to May of 2012 to 2015 (Fig. 7, left). Dense blooms of *A. catenella* in Osaka Bay occurred in 2013, 2014 and 2015, but not in 2012. The highest abundance at the two sampling locations was 833; 10,080; 452,670, and 10,821 cells L^{-1} in 2012 (May 7), 2013 (April 16), 2014 (April 15), and 2015 (March 24), respectively. The temporal variation in the relative sequence abundance of the OTU associated with *A. catenella* displayed a similar pattern with the number of cells detected by light microscopy (Fig. 7, right). Before and middle of the *A. catenella* blooms in 2013, 2014 and 2015, the community diversity of the plankton assemblage in Osaka Bay decreased based on Simpson's index (Fig. 4). The average abundance of top 30 OTUs associated with microalgae top showing the largest change in average abundance

Table 1

Meteorological and hydrobiological data taken in Kankujima station during February–April of 2012–2015. The data were downloaded from the official website of the Japan Meteorological Agency (available online from <http://www.jma.go.jp/>).

Year	Month	Precipitation (mm)	Raining day	Solar radiation time (hour)	Maximum wind speed (m s ⁻¹)	Peak gust (m s ⁻¹)
2012	February	107.5	10.0	104.4	13.6	19.0
	March	104.5	9.0	152.9	16.1	21.6
	April	79.0	11.0	200.1	24.7	31.9
	February–April	291.0	30.0	457.4		
2013	February	80.0	11.0	139.5	14.9	21.1
	March	68.5	6.0	197.0	19.4	24.7
	April	65.0	9.0	211.2	16.7	22.1
	February–April	213.5	26.0	547.7		
2014	February	48.5	7.0	112.3	11.9	15.9
	March	137.0	11.0	180.9	16.4	23.1
	April	70.0	8.0	201.3	14.3	19.0
	February–April	255.5	26.0	494.5		
2015	February	17.0	5.0	124.4	14.8	19.0
	March	157.5	11.0	163.2	14.7	23.7
	April	95.0	14.0	142.6	17.6	22.6
	February–April	269.5	30.0	430.5		
2005–2014 average (sd)		232.8 (74.0)	27.2 (4.4)			

between 2012 (no *A. catenella* bloom) and 2013–2015 (*A. catenella* blooms) indicated that at both stations the highest number of OTUs showing a decrease and increase compared to 2012 were associated with dinoflagellates, whereas second largest decrease at both stations was displayed by OTUs associated with diatoms (Fig. 8 A, B). Majority of the OTUs displaying the largest change were same at both stations, however, 9 OTUs differed between the stations (Fig. 8 A, B).

3.4. Correlations between environmental parameters and biological data

Of all the abiotic and biotic parameters investigated, statistically significant ($P < 0.05$) positive relationship was detected between the number of OTUs detected and Simpson's index, PO_4 and $\text{Si}(\text{OH})_4$, and PO_4 and DIN concentrations at both stations (Table 2). Statistically significant ($P < 0.05$) positive relationship was also detected between

Fig. 2. Changes of water temperature and salinity during the monitoring from February to May in 2012–2015 at two localities in Osaka Bay. The x-axis shows dates (day/month).

Fig. 3. Changes of dissolved inorganic nitrogen (DIN = $\text{NO}_3 + \text{NO}_2 + \text{NH}_4$), phosphate ($\text{PO}_4\text{-P}$), and silicic acid ($\text{Si(OH)}_4\text{-Si}$) concentrations during the monitoring from February to May in 2012–2015 at two localities in Osaka Bay. The x-axis shows dates (day/month).

Fig. 4. Temporal variations in community diversity indices at two localities in Osaka Bay from February to May during 2012–2015. Left, Species richness (the numbers of OTUs); Right, Simpson's index (species evenness). The x-axis shows dates (day/month).

Fig. 5. A plot of NMDS analysis of temporal patterns in 122 samples of plankton collected at two localities in Osaka Bay ($k = 3$, stress 0.17). A: Differences in biodiversity among years.; B: Differences of biodiversity among months with water temperature, salinity, DIN, PO₄, and Si(OH)₄ included as environmental vectors.

Fig. 6. A Venn diagram showing the number of shared OTUs obtained by the MPS-based monitoring in the 122 seawater samples at two localities in Osaka Bay.

Si(OH)₄ and DIN concentrations at station 12 and between *A. catenella* relative abundances detected by MPS and abundances obtained by LM at station 19. A statistically significant relationship between *A. catenella* abundances obtained by the two methods was also detected at station 12 based on the reduced dataset ($n = 26$, data not shown). No statistically significant relationships were detected between other parameters (Table 2).

4. Discussion

Alexandrium catenella blooms were detected in 2013–2015 by LM and MPS, whereas no bloom was recorded in 2012, although the presence of the species was confirmed by both methods. The plankton species diversity decreased before and middle of the *A. catenella* blooms in 2013, 2014 and 2015, potentially indicating that the presence of other species, may influence the development blooms of *A. catenella* in Osaka Bay. In general, both methods displayed a similar pattern in *A. catenella* appearance, which was further supported by a statistically significant ($P < 0.05$) correlation between the cell abundances and relative sequence read abundances.

4.1. Patterns in *A. Catenella* appearance and blooms

In Osaka Bay, *A. catenella* vegetative cells are present from February

to May (Yamamoto et al., 2017). The seasonal appearance of this species in the Seto Inland Sea is controlled by bottom and surface water temperatures: high cyst germination success at 10–15 °C (December–April; Itakura and Yamaguchi, 2001) and vegetative cells present in the water column until the surface water temperature reaches around 20 °C (Itakura et al., 2002; Yamamoto et al., 2017) with the highest encystment rate at 14 °C (Nagai et al., 2004). In this study, blooms of *A. catenella* were recorded in March and April during 2013–2015 although the species was also present in 2012, and the vegetative cells disappeared when water temperature exceeded 17 °C. Similarly to a previous study from Hiroshima Bay (Itakura et al., 2002), no statistically significant correlation could be detected between temperature and *A. catenella* cell/sequences abundances. Thus, although the temperature is an important for facilitating the germination of *A. catenella* cysts and for bloom termination, the bloom development is potentially controlled by other factors. In 2012, the wind speed and average precipitation were higher than in other years investigated, generating higher turbulence in the surface layer, that generally hampers dinoflagellate blooms (Margalef et al., 1979; Therriault et al., 1985; Uchida et al., 1998). The importance of water column stability to the development and continuation of *A. catenella* blooms has been also indicated in previous studies (Fauchot et al., 2008; Yamamoto et al., 2002; 2011; 2013; Weise et al., 2002). In addition, low spring bloom intensity due to decreased nutrient concentrations has also suggested to contribute to the occurrence of *A. catenella* blooms in the Seto Inland Sea (Itakura et al., 2002; Yamamoto et al., 2017). Unfortunately, no statistically significant relationship between nutrient concentrations and cell abundances/relative *A. catenella* sequence abundances could be detected in this study. This may be related with the lower number of nutrient samples available ($n = 26$), compared to the previous study reporting statically significant negative correlations between nutrient concentrations and *A. catenella* cell abundances (Itakura et al., 2002; $n = 67$). At the same time, the nutrient concentrations were generally lower in 2013 and 2014 (bloom) compared to 2012 (no bloom). Although in 2015 (bloom) the nutrient concentrations were generally similar or even higher than in 2012, the concentrations were reduced prior the *A. catenella* bloom, coinciding with the suggested pattern (Itakura et al., 2002; Yamamoto et al., 2017). Low nutrient levels may potentially be explained by the reduced freshwater input, e.g. lower precipitation in 2013–2014, or by the presence of large diatoms prior spring bloom, exhausting the inorganic nutrients (Nagai et al., 1996; Itakura et al., 2002).

As *A. catenella* can utilize dissolved organic phosphorous (Oh et al.,

Fig. 7. Temporal variation in abundance of *Alexandrium tamarense* at two localities in Osaka Bay from February to May during 2012–2015. Left, Cell density counted by morphology-based observations using light microscopy (cell mL^{-1}); Right, Metagenome data (relative to total abundance). The x-axis shows dates (day/month).

2002) and perform vertical migration (MacIntyre et al., 1997; Fauchot et al., 2005; Yamamoto et al., 2010), the lower nutrient concentrations observed during *A. catenella* blooms (Itakura et al., 2002) may give the species an advantage over competitors such as fast-growing diatoms (Itakura et al., 2002; Yamamoto et al., 2017; Natsuike et al., 2018). This is further supported by the reduction in average relative sequence abundances of OTUs associated with diatoms in 2013–2015 compared to 2012. Unfortunately, the change in the abundances of diatoms and dinoflagellates cannot be directly compared based on the relative sequence abundances as it may be influenced by the variations in copy number in different groups (e.g. Zhu et al., 2005; Gran-Stadniczeńko et al., 2018) and there is no phytoplankton community composition data obtained by light microscopy available from the sampling occasions. At the same time, diatom blooms generally display high species diversity (Guillard and Kilham, 1977; Smayda, 1980) compared to dinoflagellate blooms (Smayda and Reynolds, 2003) and thus the decline in species diversity detected during 2013–2015 may be regarded as a potential indicator for *A. catenella* bloom development in Osaka Bay. As a future perspective, machine-learning and an association study using data-mining to detect association rules related to the species increase and decrease based on MPS data may help to reveal patterns between species/groups abundance and diversity (e.g. *A. catenella* and diatoms) and allow prediction of blooms based on community composition data (Cordier et al., 2018; Asano et al., 2019).

4.2. Massively parallel sequencing approach for phytoplankton monitoring

Massively-parallel sequencing has become a popular method for

detecting protistan diversity and species richness in marine and freshwater ecosystems (e.g. Xiao et al., 2014; de Vargas et al., 2015). MPS technologies deliver high-throughput performance and allow the detection of several hundreds of OTUs (e.g. Tanabe et al., 2016; Nagai et al., 2016a,b; Hu et al., 2016; Elferink et al., 2017; Piredda et al., 2018). The significant advantage of applying MPS-based technology for monitoring of eukaryotic species is its great potential to identify species by genetic information, in particular, the species that are indistinguishable by morphology-based observation using LM (e.g. John et al., 2005; Lelong et al., 2012). Although, the OTU relative abundances do not reflect the number of cells directly (Egge et al., 2013; Weber and Pawlowski, 2013), they may give an overall impression of species/classes relative abundances (Giner et al., 2016; Eiler, et al. 2017; Gran-Stadniczeńko et al., 2017). In addition, when combined with LM counts, the relative sequences abundances may provide a semi-quantitative estimate of cell abundances, especially relevant for morphologically similar species, e.g. *Pseudo-nitzschia* sp. (Nagai et al., 2017). In this study, the relative sequence abundances of the OTU associated with *A. catenella* obtained by MPS displayed a similar pattern with the cell abundances from LM counts.

Interestingly, for station 12 the statistically significant ($P < 0.05$) correlation was only detected in the reduced dataset ($n = 26$). First, it was regarded to result from the rarefying procedure that randomly omits some sequencing data to normalize library sizes (McMurdie and Holmes, 2014). However, the total sequence abundances of the OTU related with *A. catenella* also did not display a statistically significant correlation with the cell abundances obtained by LM (data not shown) and thus the influence of rarefying does not explain the finding. As

A

B

Fig. 8. Changes in average relative sequence abundances of species associated with the top 30 OTUs showing highest variability between 2012 (no *A. catenella* bloom) and 2013–2014 (*A. catenella* blooms). A: station 12, B: station 19, asterisk indicates OTUs that differed among the stations, X-axis values are * 100 to improve readability, negative values indicate a decrease and positive values show an increase in 2013–2014 compared to 2012.

Table 2

Pearson correlations between environmental and biotic parameters. The first number indicates *r* values for station 12 /second number indicates values for station 19. *N* = 26 for correlations with nutrients and *n* = 60 for other parameters. Bold numbers show statistically significant correlations (*P* < 0.05) after Bonferroni correction. LM: *A. catenella* cell abundances obtained by light microscopy; MP: relative *A. catenella* sequence abundances obtained by massively parallel sequencing.

	Temperature	Salinity	LM	MPS	Nr. of OTUs	Simpson's index	PO ₄	Si(OH) ₄	DIN
Temperature		0.11/-0.13	-0.03/-0.01	0.02/-0.001	-0.18/-0.19	-0.003/-0.12	-0.19/-0.10	-0.14/0.15	-0.39/-0.44
Salinity	0.11/-0.13		-0.08/-0.11	0.12/0.04	-0.09/0.34	-0.04/0.26	-0.49/0.23	-0.52/0.33	-0.61/-0.04
LM	-0.03/-0.01	-0.08/-0.11		0.37/0.67	-0.17/-0.22	0.01/-0.17	-0.17/-0.20	0.07/-0.03	-0.12/-0.13
MPS	0.02/0.001	0.12/0.04	0.37/0.67		-0.15/-0.26	-0.03/-0.28	-0.13/-0.22	0.08/-0.06	-0.11/-0.19
Nr. of OTUs	-0.18/-0.19	-0.09/0.34	-0.17/-0.22	-0.15/-0.26		0.82/0.81	0.46/0.28	0.44/0.14	0.45/0.11
Simpson's index	-0.003/-0.12	-0.04/0.26	0.01/-0.17	-0.03/-0.28	0.82/0.81		0.38/0.19	0.45/0.04	0.34/0.01
PO ₄	-0.19/-0.10	-0.49/0.23	-0.17/-0.20	-0.13/-0.22	0.46/0.29	0.38/0.19		0.83/0.86	0.93/0.75
Si(OH) ₄	-0.14/0.15	-0.51/0.33	0.07/-0.03	0.08/-0.06	0.44/0.14	0.45/0.04	0.83/0.86		0.86/0.50
DIN	-0.39/-0.44	-0.61/-0.04	-0.12/-0.13	-0.11/-0.19	0.45/0.11	0.38/0.01	0.93/0.75	0.86/0.50	

another aspect, usage of Bonferroni correction to account for multiple testing was considered as this method is conservative and increases the likelihood of rejecting significant differences (Perneger, 1998). However, this was not the cause as other adjustments (Holm, Hochberg, Hommel, Benjamin-Hochberg) applied to the same dataset resulted also in statistically not significant result, except for Benjamin-Hochberg adjustment. Potential misidentification of *A. pacificum* as *A. catenella* by using LM was also investigated as both species are present in Osaka Bay and have a similar size (John et al., 2014), whereas other morphological features, e.g. presence/absence of a ventral pore are not observable without staining and epifluorescence. However, based on the MPS-data, OTU associated with *A. pacificum* was only detected on one occasion from station 12 and thus misidentification does not seem plausible. Thus, the potential reasons causing the lack of correlation when using the full dataset from station 12 remains currently unresolved. Nevertheless, based on plotting the relative sequence abundances of OTU related with *A. catenella* and cell abundances obtained by LM, the patterns detected by both methods display similar patterns, demonstrating the effectiveness of MPS to follow the bloom dynamics.

5. Conclusions

During four years of MPS-based monitoring, blooms of *Alexandrium catenella* occurred, when the water column was stable and the overall species diversity was reduced possibly due to low nutrient concentrations. Those factors can be used as the indices for *A. catenella* bloom occurrence to provide an early warning for local fisheries. The abundance of the toxic dinoflagellate *A. catenella* by morphology-based observation via microscopic analysis vs. the relative abundance recorded from MPS displayed similar fluctuation patterns between the two approaches, although the MPS-based method is not quantitative in principle. The comparability of two methods to detect the patterns in *A. catenella* abundances was further supported by the strong and statistically significant positive correlation.

Data accessibility

DNA sequences are available in the DDBJ Sequence Read Archive: PRJDB4580.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.hal.2019.101660>.

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