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Abstract

The relationships among eutrophication, anoxia, and microbial distribution were investigated for Nagatsura-Ura Lagoon on the northeastern Pacific coast of Japan. In September 2017, the bottom environment in a small area of the inner part of the lagoon (which has a basin-shaped bottom topology) was eutrophic and anoxic, with high carbon, nitrogen, phosphate, acid-volatile sulfide, and low dissolved oxygen and oxidation-reduction potential. Dissolved oxygen levels improved during the winter. Bacillariophyta (diatoms) were the main organic component according to pigment analysis and next-generation sequencing of nucleic acids in seawater samples. Phylum Proteobacteria was dominant among the bacterial flora in the sediment but the proportions of Class Epsilon-proteobacteria and *Chlorobium* (a green sulfur-utilizing bacterium) were high in the inner part of the lagoon compared to other stations, and these groups were also present in winter. Apparently groups able to thrive in both anoxic and aerobic conditions were predominant in the inner part of the lagoon.

Introduction

While oxygen is vital for many organisms, factors such as global warming are causing the spread of eutrophication and reducing the oxygen content of certain areas of the ocean (Breitburg et al., 2018). The sources of eutrophication in coastal areas are the nutrients supplied from rivers, agricultural fertilizers (Diaz and Rosenberg, 2008), groundwater (Van Meter et al., 2017), sewage (Breitburg et al., 2018), or elution from the seafloor (Ingall and Jahnke, 1994). The increase in primary producer biomass caused by eutrophication is responsible for increased oxygen consumption as a result of increased bacterial production (Yamamuro and Kamiya, 2014). As global warming progresses, resulting in increased stratification of the oceans, regions of hypoxia or anoxia are expected to increase (Stramma et al., 2008, Diaz and Rosenberg, 2008, Breitburg et al., 2018), so it is important to understand the chemical, microbiological, and topographical factors involved in eutrophication, anoxia and hypoxia.

Nagatsura-Ura is a basin-shaped coastal lagoon with an area of about 1.41 km^2 connected by a narrow channel to Oppa Bay, which receives the mouth of the River Shin-Kitakami (an A-class river; Figure1; Takasaki and Tanaka, 2002). The lagoon depth is about 4 m at the center and about 10m at the end furthest from the connecting channel (Okumura et al., 2021). Nutrients are supplied to the lagoon from the river (Kaneko et al., 2019), so the phytoplankton blooms for a long period during spring and summer (Kaneko and Hara, 2020). The inner part of the lagoon is used for oyster farming (Murata et al., 2021) and, more than elsewhere, is a sink for organic matter including fecal pellets and periphyton (Takasaki and Tanaka, 2002). Hypoxia or anoxia occur from summer to autumn near the seabed, though only at this inner part of the lagoon (Igarashi, 2006), but in winter and spring these anaerobic conditions are

eliminated by vertical mixing (Kaneko et al., 2019). With its complex bottom topography, and a bottom environment including eutrophication and anaerobia varying over small distances, it is of interest find out whether the microbial community composition fluctuates rapidly due to bottom topography and environmental changes, or maintains a similar community structure despite these changes?

When bacteria decompose organic matter, hypoxia or anoxia is caused by the consumption of oxygen (Yamamuro and Kamiya, 2014, Breitburg et al., 2018). To understand hypoxia- or anoxia-related bacteria, the community has been identified recently by analysis of 16SrDNA amplicons in seawater and sediment using next-generation sequencing (NGS; Choi et al., 2016, Walsh et al., 2016, Nimnoi and Pongsilp, 2020). Phytoplankton, which is a major form of organic matter composed of primary producers, can be analyzed using amplicons of 18SrDNA (Okumura et al., 2021) or Psb *A* genes (Okumura et al., 2020). The merit of applying NGS to phytoplankton is to identify microscopic organisms which cannot be identified by microscopy. Since microbial ecosystems are best understood by investigating bacteria and eukaryotes simultaneously (Ul-Hasan et al., 2019), application of NGS to both phytoplankton and bacteria enables immediate identification of the flora involved in eutrophication and hypoxia or anoxia.

Further studies at both global and regional scales are necessary in order to understand the increasing occurrences of declining oxygen concentration in various areas (Breitburg et al., 2018). Therefore, the aim of the present study was to improve our understanding of how marine environment parameters such as seabed topography, eutrophication, and dissolved oxygen affect the composition of the microbial community in aquatic sediments. During the autumn, when anoxia and hypoxia are observed near the seabed in the inner part of the lagoon, we examined the horizontal

distribution of many environment parameters and phytoplankton communities in the seawater, and bacterial communities in the sediment. In addition to characterizing each sampling site, and measuring the environmental parameters affecting the microbial community structure through statistical analysis, the effects of seasonal fluctuations in dissolved oxygen on the composition of the microbial community were also determined.

Materials and Methods

Sampling locations, observations and sample collection

The site of this study was Nagatsura-Ura Lagoon, which is deepest at the furthest point from its narrow mouth. Detailed horizontal distributions and temporal changes of microbial assemblages and environmental parameters were investigated.

The horizontal distribution of environmental parameters in sediment and water was investigated from samples were collected at 31 stations in September 2017 (Fig. 1). Note that the station identifications are not uniform but conform to those established for different purposes during several previous sampling studies. The salinity and water temperature were measured by conductivity, temperature, and depth (CTD) and dissolved oxygen (DO) was measured with a profiler (Rinko-Profiler, JFE-Advantech, Hyogo, Japan). To investigate rough time trends in these parameters, sediment samples were collected at 2 stations (Stn.B and Stn.N13) inside and outside the lagoon six times from January 2016 to July 2017. The sediment samples were collected by a hand grab sampler (Rigosha Co. Ltd., Tokyo, Japan). Samples were transported to the laboratory and stored at -20°C until analyses were performed. Surface seawater samples were collected in a bucket, and seawater from 5 m depth was collected with a Kitahara water

sampler (Rigosha Co. Ltd., Tokyo, Japan). When water depth was shallower than 5m, seawater samples were collected from 50cm above the seafloor. Seawater samples were filtered for pigment analysis and next-generation sequencing and stored at -20°C (see pigment analysis and DNA sequencing sections below for details), as for samples of bottom sediment.

To examine temporal changes in water temperature, salinity, and dissolved oxygen over time, surveys using CTD equipment were conducted at two stations outside the lagoon (Stn. B) and at the inner part of the lagoon (Stn. N13), on a total of 21 occasions from March 2016 to December 2017 and 32 occasions from February 2016 to December 2017. CTD readings were obtained for every 0.5 m increment in depth. In addition, seawater and sediment samples were collected 6 times from January 2016 to July 2017 at both stations during winter and summer, when dissolved oxygen is known to change significantly, to determine the microbial community.

Organic matter and property analysis of sediment samples

Particle distribution and median diameter (MD) of sediments were determined using a laser diffraction particle size analyzer (SALD-2300, Shimadzu corporation, Kyoto, Japan), according the manual (Shimadzu Corporation, 2023). Oxidation-Reduction Potential (ORP), total nitrogen (TN), total phosphate (TP), total organic carbon (TOC), ignition loss (IL) and mud content in sediments were determined using sediment survey method protocols (Ministry of Environment Japan, 2012). ORP was measured with a portable dissolved oxygen and pH meter (DM-32P, TOA-DKK, Tokyo, Japan) with an electrode inserted into the sediments. Sediment samples for pretreatment

of TN were dried and ground in a mortar. Sediment samples for pretreatment of TOC were ground in a mortar and carbonate was removed using hydrochloric acid. After pretreatment, TN and TOC were measured with a CHN analyzer (Microcorder-JM10, J-Science, Tokyo, Japan). The C/N ratio was defined as TOC divided by TN. TP was measured by spectrophotometry using the Molybdenum blue reaction after organic decomposition with nitric acid and perchloric acid. IL was calculated after first heating the sample at 105-110°C for 2 hours then drying the resulting sediment in a desiccator and weighing. Subsequent weight reduction by IL was measured after heating the dried sample in an electronic furnace at about 600°C for 2 hours and cooling in a desiccator for 40 minutes. Acid-volatile sulfide (AVS) in sediments was determined by detector tube (Sulphides Measurement System No330, Gastec, Kanagawa, Japan) using Water Pollution Research Guidelines (Kouseisya Kouseikaku, 1980). DO in sediment was measured with a Fluorescent Dissolved Oxygen Handy Meter (HACH HQ30d LDO101, DKK-TOA Co., Tokyo, Japan) using the Sediment Investigation and Testing Manual (Japan Sediments Management Association 2016).

Stable isotope ratio in sediments

For measurement of stable isotope ratios, samples were pretreated with 1 mol hydrochloric acid, dried at 60°C, and ground using a pestle and mortar as described previously (Sato et al., 2013). After pretreatment, stable isotope ratios for ^{13}C and ^{15}N were measured using a stable-isotope-ratio mass spectrometer (DELTA V Advantage, Therrmo Fisher Scientific).

Pigment analysis of sediment and seawater samples

Chlorophyll (Chl) was extracted from about 0.5 g sediment in 1 mL N, N-dimethylformamide (DMF) stirred with a vortex mixer for 1 min and allowed to settle for more than 24 h in a freezer at −20°C. The extract was then centrifuged at 17,000 × g for 30 min. The Chl content of the supernatant was analyzed based on an equation described previously (Omata and Murata, 1980) using an Ultraspec 3000 spectrophotometer (GE Healthcare, Little Chalfont, United Kingdom).

To quantify phytoplankton pigments, 150 ml seawater were filtered through Whatman GF/F glass microfiber filters (GE Healthcare UK Ltd., Buckinghamshire, UK). The phytoplankton pigments were extracted from the filter with 1 ml methanol and allowed to settle for 24 h in a freezer at −20°C. After the filter was removed, supernatant was collected by centrifugation at 17,000 × g for 10 min. and the pigments were analyzed by high performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) using the method of Zapata et al. (2000).

DNA sequencing and data analysis

DNA was extracted from all sediments using a kit (Dneasy powersoil kit, Qiagen, Hilden, Germany). The filter samples (0.45μm PVDF Durapore membrane, Merck, Massachusetts, United States) of 500 ml seawater suspended in 1 mL lysis buffer (Tris· HCl, pH 8.0, 5 mM EDTA, 0.3% SDS), and 200 μg/mL Proteinase K (final concentration) were vortexed and incubated at 55°C for 2 h and then at 90°C for 10 min. After the filter was removed from the lysis buffer, the liquid phase containing nucleic

178 acids was separated by centrifugation at 10,000 g for 10 min. The supernatant in the 2-
179 ml tube was preserved at −20°C until PCR.

180 The primer pair used for Psb *A* was PsbAyo1F and PsbAyo1R (Okumura and
181 Kaga, 2017) with an adapter attached outside the primer for analysis by Miseq (Illumina,
182 California, U.S.A.). The primer pair for 16S was 341F and 805R of the V3-V4 region
183 (Herlemann et al., 2011) with an adapter primer of Miseq. PCR was conducted in two
184 steps. First, primers were attached to the full length of Psb *A* primers and parts of the
185 adapter sequences for Miseq. The PCR reaction was for 2 min at 94℃ for preheating,
186 followed by 35 cycles for 10 s at 98℃, 30 s at 55℃ for annealing, 1 min. at 68℃ for
187 extension, with a final extension for 10 min. at 68℃.

188 After the 1st PCR, PCR amplicons were treated with Exosap-it for use as a
189 template for a second round of PCR. In the second PCR, primers were attached to Psb *A*
190 primers and all adapter sequences for Miseq. The PCR reaction was for 2 min. at 94℃
191 for preheating, followed by 15 cycles for 10 s at 98℃, 30 s at 55℃ for annealing, 1 min.
192 at 68℃ for extension, with a final extension for 10 min. at 68℃. KOD-Fx (Toyobo,
193 Osaka, Japan) was used as the PCR enzyme. The reagent was prepared according to the
194 KOD-Fx manual.

195 After the second round of PCR, each amplicon was subjected to electrophoresis
196 and purified from the gel (Nippon Genetics Co,. Ltd, Tokyo, Japan). After all PCR
197 amplicons were purified and combined, a 300 bp paired-end analysis was performed
198 using Miseq. After the DNA was sequenced, the sequence of each sample was assigned
199 to each adapter. 16S was also processed using the same PCR, purification and sequence
200 method as for Psb *A*. After merging and trimming the sequences, they were analyzed
201 with QIIME (Caporaso et al. 2010) using the original Psb *A* database and Greengenes
202 database (ver.13_8). All sequenced data (DRA012462) were registered in the DNA

Data Bank of Japan.

Horizontal maps and changes over time of environmental parameters and microbial assemblages

In September 2017, Horizontal distributions of DO, Chl *a*, Fucoxanthin (Fuco), ORP, MD, AVS, IL, TOC, TN, TP, C/N, $\delta^{13}C$, $\delta^{15}N$, 16S rRNA gene, and Psb *A* in Nagatsura-Ura Lagoon were drawn with Surfer 15 (Golden Software LLC, Colorado, USA) under default parameter values using the survey data. Contour figures of horizontal distribution were combined with the survey data and map data from the national land numerical information download service (http://nlftp.mlit.go.jp/ksj-e/index.html), using the Gun Image Manipulation Program (Gimp ver.2.10; https://www.gimp.org/).

To examine changes in DO, water temperature, and salinity over time in Stn. N13 at the inner part of the lagoon from February 2016 to December 2017, contour plots were created from CTD results using ODV's Data-Interpolating Variational Analysis (DIVA) (Troupin et al., 2012). For comparison, contour plots were also made at Stn. B outside the lagoon. The changes of microbial assemblages over time were calculated using Microsoft Excel.

Statistical analysis: redundancy analysis (RDA)

To understand the relationship between the bacterial communities and the environmental parameters in the sediment samples, redundancy analysis (RDA) was conducted using Canoco (http://www.canoco5.com/), for all environmental parameters

in all 31stations in September 2017 and bacterial composition obtained by NGS, according to the manufacturer's protocol.

Results

Dissolved oxygen (DO), Chlorophyll *a* (Chl *a*) and Fucoxanthin (Fuco) concentrations in seawater

In comparison with DO values in surface seawater, DO in deeper layers varied greatly across the stations in September 2017 (Figure 2a, d). DO in surface seawater ranged from 8.0 to 9.3 (mean 8.6) mg/L; at a depth of 50 cm above the bottom it ranged from 0.04 to 9.0 (mean 5.3) mg/L; and the inner part of the lagoon in particular showed lower values than other stations. Conversely, DO on the east side was higher than that outside the lagoon.

Chl *a* and Fuco concentrations in surface seawater were higher in the lagoon than outside, and their horizontal distributions were similar (Figure 2b, c). Chl *a* concentrations ranged from 1.1 to 16 (mean 6.8) µg/L (Figure 2b). Fuco concentrations ranged from 0.2 to 4.2 (mean 1.2) µg/L (Figure 2c). The horizontal distributions of Chl *a* and Fuco were similar in the lower layer (Figure 2e, f), comparable to those at the surface (Figure 2a,b). Chl *a* concentration in the lower layer ranged from 1.7 to 17.3 (mean 6.7) µg/L (Figure 2e) and Fuco concentrations ranged from 0.3 to 4.3 (mean 1.5) µg/L (Figure 2f). Although the stations where maximum Chl *a* and Fuco concentrations occurred in the lower layer were different from those at the surface, their horizontal distribution in lower layers was similar to those at the surface (Figure 2b, c, e ,f).

Horizontal distribution of DO, ORP, MD, AVS, IL, TOC, TN, TP, Chl *a*, C/N, $\delta^{13}C$, and $\delta^{15}N$ in sediments

The horizontal distribution of DO and ORP varied greatly among the stations (Figure 3, Supplemental Table 1). DO ranged from 0 to 8.8 (mean 4.7) mg/L and ORP ranged from -236 to 294 (mean 128) mV. Both were low in the inner part of lagoon. MD tended to be lower in the lagoon than outside the lagoon, ranging from 7.4 to 56.5 (mean 17.6). AVS, IL, TOC, TN, and TP were high in the inner part of lagoon. AVS ranged from <0.01 to 12.5 (mean 1.9) mg/g. IL ranged from 22.8 to 60 (mean 54.1) %. TOC ranged from 0.3 to 46.2 (mean 15.1) mgC/g. TN ranged from 0.04 to 4.7 (mean 1.7) mgN/g. TP ranged from 0.16 to 1.2 (mean 0.5) mgP/g. Chl *a* was higher in the lagoon than outside the lagoon, the concentrations ranged from 0.01 to 4.6 (mean 2.3) mg/g. C/N was higher in eastside of the lagoon, ratios ranged from 6.3 to 15.2 (mean 9.1). $\delta^{13}C$ and $\delta^{15}N$ did not show clear characterization in comparison with the other parameters, but $\delta^{13}C$ tended to be high at the inner end of the channel into the lagoon, and $\delta^{15}N$ was higher at the outer end of the channel. $\delta^{13}C$ ranged from -24.6 to -21.1 (mean -23.3) ‰. $\delta^{15}N$ ranged from 3.1 to 5.4 (mean 4.3) ‰.

Identification of taxon groups in seawater and sediment by amplicon sequencing of the Psb *A* gene

In the seawater in September 2017, diatoms (Bacillariophyta) of Class Coscinodiscophyceae were the predominant taxon in all stations (Figure 4a). The percentages of Coscinodiscophyceae in all sequence reads ranged from 30.5 to 87.6%

(mean 64.6%) in surface seawater, and 21.9 to 99.3% (mean 83.3%) in the lower layer. Coscinodiscophyceae tended to comprise a higher percentage in samples from the the lower layer than those at the surface. The dominant diatoms in this class were the species *Cyclotella meneghiniana*, *Minidiscus trioculatus*, *Thalassiosira pseudonana*, and *Thalassiosira_punctigera* (Supplemental Figure 1). Non-diatom taxa comprising more than 10% of organisms detected in the surface samples were 10.5% Mediophyceae in Stn.K, 35.7% Chlorodendrophyceae, of which *Tetraselmis marina* and *Tetraselmis suecica* are predominant, in Stn.1, 34.7% of Trebouxiophyceae, which *Picochlorum* sp. was predominant, in Stn.N8, 23% of Coccolithophyceae, which *Phaeocystis* sp. was predominant, in Stn.B, 32.9% of Dinophyceae, which *Akashiwo sanguinea* was predominant, in Stn.17, 12.5% of Raphidophyceae, which *Heterosigma akashiwo* was predominant, in Stn.13. From 5m depth, Trebouxiophyceae was 43.8% in Stn.1.

The Coscinodiscophyceae diatoms also dominated the lagoon sediments in September 2017, ranging from 7.3 to 90.2% (mean 64%) in all samples (Figure 4b). Sequences from Class Raphidophyceae tended be present in higher numbers near the river (Stn.A) and lagoon mouth (Stn.13), ranging from 0.1 to 75.5% (mean 11.5%) of all sequences detected. Taxa with locally high proportions (over 10%) were 57.1% Chrysomerophyceae in Stn.K, 18.4% Trebouxiophyceae in Stn.31, and 10% Dictyochophyceae in Stn.B.

From January 2016 to July 2017, Coscinodiscophyceae was also the predominant taxon in both the inner part of the lagoon (85 to 93.3%, mean 89.7%; Fig. 4c) and outside the lagoon (11.6 to 97.8%, mean 64.4%). In July 2017, Coscinodiscophyceae was less common and Raphidophyceae predominated (72.6% of all organisms detected).

Identification of taxon groups from sediment in September 2017 by amplicon sequencing of the 16S rRNA gene

Considering the bacterial flora, Phylum Proteobacteria was predominant (41.0 to 94.6%, mean 58%). However, the pattern of horizontal distribution and quantity detected varied with the class of Phylum Proteobacteria. Alpha-proteobacteria tended to be high in the mouth of the lagoon, and ranged from 2 to 15.2 (mean 5.7) % (Figure5a, Supplemental Figure 2). Deltaproteobacteria tended to be high in the lagoon, and ranged from 1.3 to 27.3 (mean 18.9) % (Figure5b, Supplemental Figure 2). Epsilon-proteobacteria tended to be high in the inner part of lagoon, and ranged from 0 to 16.2 (mean 3.8) % (Figure5c, Supplemental Figure 2). Gamma-protepbacteria tended to be high outside of the lagoon, and ranged from 5.3 to 86.8 (mean 29) % (Figure5d, Supplemental Figure 2).

Bacteria other than Proteobacteria also showed different trends in horizontal distribution. Phylum Actinobacteria tended to be high in the river mouth (0.7 to 24.5%, mean 5.9%; Figure5e, Supplemental Figure 3). Phylum Bacteroidetes, which was the second most common, tended to be high in the estuary and the lagoon (0 to 23.9%, mean 12.5%; Figure 5f, Supplemental Figure 3). Phylum Chlorobi showed a similar distribution trend to Epsilon-proteobacteria, being more common in the inner part of the lagoon (0 to 35.6%, mean 4.5%; Figure 5g, Supplemental Figure 3). The eastern and the inner parts of the lagoon appeared to have a high proportion of Phylum Cyanobacteria (0 to 10.9%, mean 3.1%; Figure 5h, Supplemental Figure 3) but most of this was chloroplast DNA of Stramenopiles (Supplemental Figure 4 and 5).

Statistical analysis by RDA of the sediment samples in September 2017

RDA was performed for the major bacterial taxa and environmental parameters (Figure 6). Some bacterial taxa showed apparent alignment with environmental parameter vectors: those of TN, TP, TOC, AVS, IL, and mud fraction <0.075 mm were in the same direction and aligned with the vectors of bacterial groups Chlorobi and Epsilon-proteobacteria. Although there was no clear relationship between the vector of Delta-proteobacteria and environmental factors, it showed similarities with the vectors of Epsilon-proteobacteria, Chlorobi, and Bacteroidetes. The vectors of ORP and DO were aligned in the opposite direction, associated with those of Alpha-proteobacteria and Actinobacteria. The direction of the vector of Gamma-proteobacteria also showed some association with Alpha-proteobacteria, but to a lesser extent. The vector magnitude of δ13C was the shortest among all the vectors, but its vector angle was found to be most closely related to Gamma-proteobacteria.

DO, temperature, and salinity from February 2016 to December 2017

The environment in Stn. N13 at the inner part of the lagoon was different from Stn. B outside the lagoon (Fig. 7). DO values ranged from 0.8 to 14.6 mg/L in Stn.N13 and 5.6 to 12.1 mg/L in Stn.B, and tended to be high from autumn to spring and low from spring to autumn. DO values in the inner part of the lagoon fluctuated more widely than outside, and were at hypoxic levels from late spring to autumn. In the inner part of the lagoon, DO was constant throughout the surface to lower layers because of vertical mixing during winter (Figure 7a), but became anoxic in the lower layers from late

353 spring to autumn as water temperatures increased and a thermocline formed from late
354 spring. Therefore, DO in the lower layers in the inner part of the lagoon varied greatly
355 depending on the season. Water temperature ranged from 5.2 to 26.3°C in Stn.N13 and
356 from 9.4 to 26°C in Stn.B, and water temperature in winter in the inner part of the
357 lagoon tended to be lower than outside. Salinity ranged from 14 to 33.4 PSU in Stn.N13,
358 and from 6.0 to 34 PSU in Stn.B. Salinity in the outer surface layer tended to be low
359 from spring to summer.
360
361
362 **Sample composition of the bacterial community from January 2016 to July 2017**
363
364 Proteobacteria were the predominant bacterial phylum both inside and outside the
365 lagoon throughout the sampling period (Figure 8a), ranging from 33 to 52% (mean
366 42.7%) in the inner part of lagoon, and 48 to 82% (mean 60.6%) outside the lagoon.
367 However, the class composition differed between the inner and outer lagoons (Figure
368 8b): γ-Proteobacteria were more prevalent outside the lagoon (a range of 32.6 to 65.6%;
369 mean 45.5%) and δ-Proteobacteria dominating the inner part of the lagoon (14.2 to
370 21.8%; mean 18.5%). Epsilon-proteobacteria were less abundant (2 to 5.4%; mean
371 3.3%) but also in higher abundance in the inner part of the lagoon than outside it.
372 Other than Proteobacteria, in the inner part of the lagoon there were relatively
373 high numbers of Chlorobi (7.0 to 28.8%; mean 19.1%) and Bacteroides (9.6 to 13.0%;
374 mean 11.2%; Figure 8a). Cyanobacteria-like sequences ranged from 8.5 to 18.8% (mean
375 13.6%), but most sequences were from the chloroplasts of Stramenopiles, not
376 Cyanobacteria (Supplemental Figure 5).
377 Outside the lagoon, Actinobacteria was the second most common taxon, ranging

from 12.8 to 20.4% (mean 17.5%). Although there were fluctuations in the numbers of taxa detected, the change of bacterial composition was more influenced by geographical characteristics, such as occurrence in the inner part of the lagoon or outside it, rather than changes over time.

Discussion

Nagatsura-Ura Lagoon is small in size (Takasaki and Tanaka, 2002) but the seafloor topography is mortar-shaped in the inner part of the lagoon (Figure 1). As the water depth varies greatly even over short distances, the environment also varies greatly from place to place (Figure 3). DO differed greatly even over small differences in location (Figure 2a, 2d, and 3a), and in the lower layer of seawater showed a gradient with its highest levels in the northern to and eastern part of the lagoon (Figure 2d). As eelgrass beds are common in the northern and eastern parts of the lagoon (Murata et al., 2021), it was considered that this pattern may be explained by oxygen production from eelgrass photosynthesis. However, DO was low in the inner part of the lagoon (Figure 2d) because there it is basin-shaped (Figure 1, this paper; see also Okumura et al., 2021) and seawater exchange between the upper and lower layers is restricted by a thermocline during the summer and autumn (Kaneko and Hara, 2020), rendering the lower layer hypoxic in the inner part of the lagoon (Figure 2d). The environmental differences among the different locations were more apparent in the bottom sediment than in the water column: DO and ORP were both low in the bottom sediment in the inner part of the lagoon (Figure 3).

MD was high outside the lagoon (Figure 3). Nagatsura-Ura Lagoon is on a rias

403 coast and the lagoon mouth is narrow (Figure 1), so the physical conditions are calm in
404 the inside but outside the conditions are transient with disruptive features such as river
405 currents. Other parameters, such as AVS, IL, TOC, TN, and TP, were high in the inner
406 part of the lagoon (Figure 3) because seawater exchange is weak there (Kaneko and
407 Hara, 2020), so it is difficult for organic matter to flow out. Moreover, suspended
408 particles are easily deposited on the substrate, such as fecal pellets from cultivated
409 oysters (Kusuki, 1977).

410 In order to understand the extent of eutrophication in Nagatsura-Ura Lagoon,
411 these environmental parameters were compared with Matsushima Bay (an enclosed bay)
412 and Sendai Bay (open), which are in adjacent parts of the coast in the same prefecture.
413 The AVS maximum was 12.5 (mean 1.9) mg/g (Figure 3, Supplemental Table 1), which
414 is much higher than the 1.48 mg/g maximum in Matsushima Bay (Oota et al., 2017). IL
415 values, which ranged from 22.8 to 90% (mean 54.1%; Figure 3), were also far higher
416 than those found in Matsushima Bay (2.6 to 15.3%; mean 9.6%; Oota et al., 2017). The
417 amount of organic matter in Nagatsura-Ura Lagoon was much higher than that in
418 Matsushima Bay, suggesting that eutrophication is in progress in Nagatsura-Ura Lagoon.
419 TOC and TN ranged from 0.3 to 16.2 (mean 15.1) mgC/g (Figure 3), and from 0.04 to
420 4.7 (mean 1.7) mgN/g (Figure 3, Supplemental Table 1). In Sendai Bay, TOC and TN
421 ranged from 0.2 to 53.15 (mean 10.9) mgC/g, and 0.01 to 3.95 (mean 1.0) mgN/g,
422 respectively (Gambe et al., 2015). TOC and TN in the lagoon were less variable than in
423 Sendai Bay, but the mean values were higher.

424 C/N ratios in the lagoon varied according to station (Figure 3, Supplemental Table
425 1): highest (15.2) in the eastern part of the lagoon, where there is much eelgrass,
426 *Zostera japonica*. The C/N ratio of *Z. japonica* is reported to be above 14.2 (Yamamuro
427 and Kamiya, 2014), so the C/N ratio in the east of the lagoon appears to be affected by

the presence of *Z. japonica*. The C/N ratio was lower (8.5) in the inner part of lagoon (Figure 3, Supplemental Table 1). The mean C/N ratio of bacteria is reported to be between 5 and 7 (Fukuda et al., 1998), and <10 for algae (Lamb et al., 2006), values that seem to match those in the presence of phytoplankton deposition and high bacteria content. Higher values for other parameters, such as TOC and TN, in the inner part of the lagoon (Figure 3) appear to be explained by the large quantities of nutrients supplied from the Shin-Kitakami River especially with the prevailing northeasterly wind from March to September (Kaneko and Hara, 2020) in association with seabed close by, oyster cultivation, the establishment of a thermocline, and low water exchange via the narrow lagoon mouth.

Among all stations monitored, $\delta^{13}C$ ranged from -24.6 to -21.1‰ (mean -23.3‰; Figure 3), and $\delta^{15}N$ from 3.1 to 5.4‰ (mean 4.3‰; Figure 3, Supplemental Table 1). $\delta^{13}C$ and $\delta^{15}N$ in surface sediments at Sendai Bay ranged from -26.5 to -19.7 (mean -22.2) ‰, and from 1.1 to 9.0 (mean 4.8) ‰ (Gambe et al., 2015). $\delta^{13}C$ and $\delta^{15}N$ in sediment cores at Onagawa Bay, Miyagi ranged from -24.3 to -21.9‰, and from 5.1 to 5.9‰ (Okumura et al., 2020). The values of $\delta^{13}C$ and $\delta^{15}N$ were resemble to the other coasts at Miyagi Prefecture. Particle organic carbon (POC) is generally related to phytoplankton (Yamaguchi et al., 2003), $\delta^{13}C$ range from -30 to -25 ‰ in freshwater phytoplankton (Meyers, 1994, Lamb et al., 2006), and from -23 to -16 ‰ in marine phytoplankton (Meyers, 1994, Lamb et al., 2006). $\delta^{13}C$ of phytoplankton in seawater is higher than that in freshwater (Lamb et al., 2006), and the $\delta^{13}C$ values of the sediments in the lagoon were within the range for marine phytoplankton, so many of the stations were clearly under a strong marine influence. $\delta^{15}N$ ranges between 3 and 12‰ for marine phytoplankton in temperate zones, and is around 5‰ for freshwater phytoplankton (Maksymowska et al., 2000). $\delta^{15}N$ values in Nagatsura-Ura Lagoon were

453 within the range for both marine and freshwater.

454 The inner part of the lagoon is eutrophic since values of IL, TOC, TN, and TP
455 were high (Figure 3, Supplemental Table 1). A major factor contributing to
456 eutrophication may be the presence of a large biomass of marine phytoplankton, as
457 shown by the high Chl *a* values (Figure 2b, 2e, and 3), C/N range, and $\delta^{13}C$ values
458 (Figure 3). Chl *a* and Fuco of phytoplankton pigments were at higher levels in the
459 lagoon than outside (Figure 2b, 2c, 2e, and 2f). The horizontal distribution patterns of
460 both Chl *a* and Fuco were similar, suggesting that certain phytoplankton, many of which
461 have Fuco, were predominant. Phytoplankton pigments differ according to taxon
462 (Jeffrey and Vesk, 1997) and -Bacillariophyta, Prymnesiophyceae, Chrysophyceae, and
463 Raphidophyceae, which have Fuco (Jeffrey and Vesk, 1997), were predominant in
464 Nagatsura-Ura Lagoon, according to pigment analysis. From the NGS data, the
465 occurrence of members of the Coscinodiscophyceae was high (Figure 4a and b),
466 suggesting that Bacillariophyta were predominant in the lagoon phytoplankton in
467 September 2017.

468 Water flow is complex and intense near a river mouth, so the phytoplankton
469 assemblages near Nagatsura-Ura Lagoon probably changed during the investigation
470 period. Raphidophyta or Cryptophyta are known to temporarily increase in this lagoon
471 with environmental changes such as increasing temperature (Kaneko and Hara, 2020)
472 but Bacillariophyta are reported to be predominant in nearby localities such as Sendai
473 Bay (Watanabe et al., 2017), Onagawa Bay (Masuda et al. 2021), and Ofunato Bay
474 (Okumura and Kaga, 2017). Therefore, the predominance of Bacillariophyta is
475 characteristic of the coastal areas of the northeastern Pacific coast of Honshu. Rivers are
476 a source of silica (Treguer et al., 1995), which is necessary for the growth of
477 Bacillariophyta, so since Nagatsura-Ura Lagoon is located at a river mouth (Figure 1),

it should be an ideal environment for the growth of Bacillariophyta. Although phytoplankton composition differed slightly between seawater and sediment, Bacillariophyta was the dominant taxon in both (Figs. 2c, 2f, 4). Phytoplankton in seawater falls to the seabed by gravity, either directly or as fecal matter from filter feeders such as oysters. Therefore, Bacillariophyta (predominant in seawater) were assumed to be predominant also in the sediment. Phytoplankton data for seawater are instantaneous results for the investigation date, whereas phytoplankton data from sediment is an accumulation of phytoplankton deposited over a period of time. Slight differences in the floral composition of seawater and sediment are therefore not unexpected. As a major source of organic matter, Bacillariophyta are thought to contribute to eutrophication in the inner part of Nagatsura-Ura Lagoon.

From the above results, the mechanism of eutrophication in Nagatsura-Ura Lagoon is considered to be as follows: 1) Nutrient-rich water from Shin-Kitakami River flows into Nagatsura-Ura Lagoon; 2) Phytoplankton grow in the nutrient-rich seawater there; 3) Phytoplankton are mostly deposited on the seabed either directly or in the fecal pellets of oysters; 4) As the inner part of the lagoon is mortar-shaped, organic matter on the seabed exits the lagoon with difficulty due to the low degree of seawater exchange near the bottom, especially from summer to autumn when the water mass structure becomes stratified.

The composition of bacteria communities in the sediments (Figures 4, 5) also differed (Figure 6) according to the environment at the sampling sites (Figure 3). DO was low in the lower water layer and in the sediment of the inner part of the lagoon (Figure 2d, and 3a). The decrease of water exchange and the increase of organic matter in the sediment are thought to accelerate the consumption of oxygen due to bacterial decomposition, resulting in hypoxia or anoxia (Yamamuro and Kamiya, 2014). The

503 components of bacterial communities are known to vary with locality and environment
504 (Hamdan et al., 2013, Walsh et al., 2016), so it is no surprise that the bacterial
505 community in the sediments around Nagatsura-Ura Lagoon differed at each sampling
506 station (Figure 5 and 7). Around Nagatsura-Ura Lagoon, Phylum Proteobacteria was
507 predominant, followed by Bacteroidetes (Figure 5a to d, f, and 7), both of which are
508 known to be abundant in aquatic environments (Cottrell and Kirchman, 2000,
509 Kirchman, 2002, Stevens et al., 2005, Nimnoi and Pongsilp, 2020, Choi et al., 2016).
510 Within Phylum Proteobacteria, Alpha-proteobacteria tended to dominate just
511 outside the mouth of the lagoon (Figure 5a), and were correlated with DO and ORP in
512 the RDA analysis (Figure 6). Alpha-proteobacteria are more tolerant to low rather than
513 high nutrient concentrations (Pinhassi and Berman, 2003, Dai et al., 2013), which is
514 consistent with their abundance at sites of lower nutrient concentrations and being less
515 common in the inner part of lagoon.
516 Gamma-proteobacteria, too, tended to be more abundant outside the lagoon,
517 which is less eutrophic and hypoxic than the inner part of lagoon (Figure 5d). In the
518 RDA, Gamma-proteobacteria were related to DO and ORP, as well as Alpha-
519 proteobacteria (Figure 6). Although its vector magnitude is short, the $\delta^{13}C$ vector angle
520 was closely aligned with that of Gamma-proteobacteria, rather than Alpha-
521 proteobacteria. The reason for this association is considered to be that Gamma-
522 proteobacteria were detected closer to Oppa Bay than the river, which is higher in
523 Alpha-proteobacteria (Figure 5d). Indeed, they are halophilic (Wu et al., 2006) and
524 considered to be abundant in the oceans (Dai et al., 2013), being predominant in the
525 North Sea (Eilers et al., 2000) and certain marine sediments (Franco et al., 2017).
526 Phylum Bacteroidetes was second in predominance after Phylum Proteobacteria
527 (sum of Alpha-, Delta-, Epsilon-, and Gamma-) around Nagatsura-Ura Lagoon in

September 2017 (Figure 5). It has been found to be abundant in freshwater lakes (Dai et al., 2013) and has also been detected in anoxic conditions which include hydrogen sulfide (Kondo et al., 2009). Members of the Cytophaga-Flavobacteria cluster of Phylum Bacteroidetes are most abundant in sediment layers, followed by the sulfate-reducing bacteria (Llobet-Brossa et al., 1998) and was consistent with finding a large proportion of Bacteroidetes genes at the mouth of the Shin-Kitakami River and in the inner part of the lagoon.

Phylum Actinobacteria, which tended to be most abundant near the river mouth (Figure 5e), is common in freshwater (Nimnoi and Pongsilp, 2020) and in South Korea and this group has been recorded as the next most abundant bacteria after Proteobacteria and Bacteroidetes in the bottom mud of Jeju Island (Choi et al., 2016), and Incheon tidal flats (Choi et al., 2018). The growth environment of Actinobacteria near Nagatsura-Ura Lagoon matches these past findings.

DO in the inner part of the lagoon showed large seasonal changes (Figure 7a) but the bacterial community showed little difference through winter, spring and summer (Figure 8), and autumn (Figure 5). In the basin of the inner part of the lagoon (Figure 1), DO in the lower seawater layer and in the sediment is low not only in September (Figures. 2d, 3a) but also in late spring and autumn, although hypoxia is absent from winter to spring (Figure 7a). Annual seasonal changes of DO are observed every year (Kaneko et al., 2019). Delta-proteobacteria, Epsilon-proteobacteria and Chlorobi in the inner part of the lagoon tended to be high in comparison with the outer part, regardless of season (Figure 8). In the RDA, Epsilon-proteobacteria and Chlorobi were closely associated with high values of AVS, IL, TOC, TN, and TP (Figure 6), suggesting that these bacteria contribute to eutrophication of the inner part of the lagoon. Epsilon-proteobacteria, in particular, can live in a variety environments, including aerobic,

553 microaerobic, or anoxic conditions, and are highly sulfide tolerant (Keller et al., 2015),
554 so it is speculated that their levels in the inner part of lagoon may show little change
555 over time. Chlorobi also inhabit anoxic environments, including those with high levels
556 of hydrogen sulfide (Kondo et al., 2009, Thompson et al., 2017), and some species are
557 adapted to low light levels (Overmann et al., 1992, Thompson et al., 2017). Since
558 Chlorobi (like Delta-proteobacteria and Epsilon-proteobacteria) were found in both the
559 presence and absence of anoxia, they, too, may be observed in the inner part of the
560 lagoon with little change over time.

561 While no clear relationship was observed between the proportion of Delta-
562 proteobacteria and environmental factors (Figure 6), its vector angle resembled those of
563 Epsilon-proteobacteria, Chlorobi, and Bacteroidetes, and was opposite in direction to
564 those of Alpha-proteobacteria and Gamma-proteobacteria. Therefore, it was to be
565 expected that Delta-proteobacteria would be detected in the inner part of the lagoon,
566 which is the location of oyster farming and associated eutrophication. Indeed, Delta-
567 proteobacteria include sulfate-reducing bacteria (Wang et al., 2012), are dominant in
568 anoxic environments (Bowman and McCuaig, 2003, Dai et al., 2013), and in
569 aquaculture sites in northeastern Japan (Asami et al., 2005).

570 In conclusion, diatoms were the major source of organic matter in Nagatsura-Ura
571 Lagoon, parts of which are anoxic. The sediments were eutrophic (high in nutrients and
572 AVS) only in the basin-shaped inner part of the lagoon, and the bacterial community in
573 the sediments varied among stations depending on several environmental parameters,
574 such as TN, TOC, AVS, TP, ORP, DO. Even in the restricted space of this lagoon (1.41
575 km^2), if the seafloor topography differs greatly, environmental conditions also differ
576 greatly. The difference in environmental conditions is apparently reflected in differences
577 in the bacterial communities across the small distances among sampling stations.

578 Considering seasonal changes of DO in the inner part of the lagoon, the near bottom
579 layer is anoxic from late spring to autumn, but not during winter and early spring, so the
580 seasonal fluctuation of DO was large. However, bacterial composition changed little
581 from January 2016 to July 2017 regardless of the oxygen status. Therefore bacterial
582 species which thrive during eutrophication and can cope with both aerobic and
583 anaerobic conditions in the inner part of the lagoon are dominant, and considered to be
584 largely responsible for the occurrence of the anoxia observed. This study clarified the
585 effects of the special seafloor topography (mortar shape) of the lagoon on the
586 sedimentary environment and the microbial assemblages that inhabits it.
587
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803

804 **Figure captions**

805

806 **Figure 1.** Sampling sites (marked axes are grid coordinates). The seafloor map is based
807 on Okumura et al. (2021). Color bar indicates water depth of seabed.

808

809 **Figure 2.** Horizontal distributions of dissolved oxygen (DO), Chlorophyll *a* (Chl *a*), and
810 Fucoxanthin (Fuco) concentrations in seawater. (a-c) at the surface, (d-f) at 50cm above
811 bottom.

812 **Figure 3.** Horizontal distributions of various parameters in sediments.

813

814 **Figure 4.** Identification of major diatom taxon groups by amplicon sequencing of the
815 Psb *A* gene in samples from Nagatsura-Ura Lagoon. (a) Seawater samples of surface
816 and 5 m depth in September 2017. Stations from N33 northwards are outside the lagoon.
817 Station D is at the entrance of the lagoon. Stations southwards from K are inside the
818 lagoon (refer to Fig. 1). (b) Sediment samples analyzed in September 2017. Stn.A is
819 outside the lagoon. (c) Sediment sample composition by major groups at inside the
820 lagoon (Stn. N13) and outside the lagoon (Stn. B), from Jan., 2016, to July, 2017.

821

822 **Figure 5.** Horizontal distributions of major taxon groups in September 2017 by
823 amplicon sequencing of the 16S rRNA gene. (a) Alpha-proteobacteria, (b) Delta-
824 proteobacteria, (c) Epsilon-proteobacteria, (d) Gamma-proteobacteria, (e)
825 Actinobacteria, (f) Bacteroidetes, (g) Chlorobi, (h) Cyanobacteria. Left scale gives map
826 reference northings; colors indicate percentage occurrence.

827

Figure 6. RDA of bacterial communities and environmental parameters by Canoco. (*Actinobc*, Actinobacteria; *Alphaprt*, Alpha-proteobacteria; *Betaprot*, Beta-proteobacteria; *Bacteroi*, Bacteroidetes; *Chlorobi*, Chlorobi; *Cyanobac*, Cyanobacteria; *Deltaprt*, Delta-proteobacteria; *Epsilonp*, Epsilon-proteobacteria; *Gammaprt*, Gamma-proteobacteria; *OthrBact*, Other Bacteria; *OthrProt*, Other Proteobacteria).

Figure 7. Contour graphs of DO, temperature, and salinity in Stn. N13 in the inner part of the lagoon (a), and Stn. B outside the lagoon (b), from 2016 to 2017

Figure 8. Sample composition of bacterial communities from sediments in Stn. N13 and Stn. B from Jan., 2016, to July, 2017. (a) All bacteria, (b) Proteobacteria.

Supplemental Figure 1. Identification of major phytoplankton species by amplicon sequencing of the Psb *A* gene in seawater samples from Nagatsura-Ura Lagoon in September 2017.

Supplemental Figure 2. Occurrence (percent of total) of proteobacteria in September 2017 by amplicon sequencing of the 16S rRNA gene.

Supplemental Figure 3. Occurrence of bacteria (other than proteobacteria) in September 2017 by amplicon sequencing of the 16S rRNA gene.

Supplemental Figure 4. Occurrence of cyanobacteria-related sequences in September 2017 by amplicon sequencing of the 16S rRNA gene.

853 Supplemental Figure 5. Occurrence of cyanobacteria-related sequences from Jan., 2016,
854 to July, 2017 by amplicon sequencing of the 16S rRNA gene in Stn.N13 (inner part of
855 the lagoon) and Stn.B (outer part of the lagoon).