STUDY ON A MONITORING METHOD FOR SEAGRASS BEDS USING ENVIRONMENTAL DNA

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ABSTRACT

Environmental DNA (eDNA) analysis allows information to be obtained about the identification of aquatic species. Seagrass DNA can be detected in sampled water which contains exocrine secretions from the fine pores on the leaf surface. We developed a seagrass monitoring method based on the eDNA analysis. The results show that the concentration of eDNA corresponds to the shoot density and heights of seagrass in a mesocosm pool in which seagrass was reared for more than 15 years. Seagrass DNA was exported from the seagrass beds and was transported by currents in the bay. The currents were calculated using a numerical simulation, allowing advection routes of seagrass DNA to be assigned. A large concentration of seagrass DNA in the water sampled on the advection route means that there is a lot of seagrass at the starting point. Based on this, we are developing a method to monitor the growth of seagrass at a distance by analyzing eDNA in water sampled at appropriate locations and times. The feasibility of this monitoring method is confirmed by numerical simulations of a simple bay model and a modeling of Ago bay. The characteristics of eDNA were examined in laboratory tests to increase the accuracy of analysis of DNA concentrations in sea water sampled. Our study indicates that detecting eDNA that has been transported by currents for several days is difficult because seagrass DNA in water naturally deteriorates in around five days.

Keywords: Environmental DNA, seagrass, aquatic species monitoring, particle tracking calculation, Ago bay

1. INTRODUCTION

Environmental DNA (eDNA) has attracted attention as a method for investigating aquatic organisms. Analysis of DNA derived from tissue fragments and excrement contained in water samples can yield useful biological information. Conventional biological survey is specialized mainly by divers, making it difficult to conduct frequent and extensive surveys. A monitoring method using eDNA to obtain biological information from water samples and provide more detailed information reduces the effort associated with traditional visual inspections, such as by divers.

A biological survey has been proposed to monitor seagrass beds in combination eDNA with numerical analysis in the actual sea area (Akatsuka et al., 2018). This method uses numerical simulation to predict the range of eDNA diffusion from habitats and examines the range of DNA detection. However, to monitor aquatic organisms from changes in eDNA, it is necessary to understand the amount of eDNA and from where it came from at the time of sampling. Particle tracking calculation is a method to determine the starting point of eDNA and water sampling point, and a biological monitoring method combined with eDNA analysis has been proposed (Takayama et al., 2019). This monitoring method uses numerical simulation to predict eDNA pathways arising from the habitats of target aquatic organisms that do not move in the water, such as seagrass or corals, and captures changes (such as decreases) in the target organisms by taking water samples along the pathways. If

eDNA with different points of origin do not coexist, a monitoring regime can be established by tracking changes in the amount of eDNA in samples obtained regularly at a fixed point on the predicted route.

The temporal variations in eDNA concentrations are assumed to capture seasonal changes in biomass and other sudden changes (such as dying). Although there is no report on the eDNA amount of seagrass associated with changes in biomass in the sea, the amount of eDNA varies not only with the amount of biomass but also with plant conditions such as dying and germination, physical conditions such as water depth, water temperature, water quality, flow rate, and the presence of microorganisms that degrade eDNA. It is necessary to understand the relationship between eDNA amount and seagrass biomass and the effects of other factors for monitoring changes in biomass using eDNA.

2. STUDY CONCEPT

2.1 Study purpose

The purpose of this study was to examine the feasibility of the monitoring method using eDNA from both aspects of eDNA properties and pathway prediction using numerical simulation. First, we investigated the feasibility of eDNA analysis for monitoring seasonal changes in the biomass of *Zostera marina*, a common seagrass in Japanese habitats. Secondly, eDNA monitoring could be applied at fixed point depending on the combination of seagrass bed and flow by numerical simulation using simplified bay model. Finally, eDNA analysis was attempted in Ago bay as an actual sea and summarizing the future subject for an actual sea survey.

2.2 eDNA analysis using the water of a mesocosm experimental tank

We examined eDNA properties about the particle size, the degradation process, and the relationship between biomass and eDNA concentrations. These experiments were performed in a mesocosm experimental tank that *Z. marina* grew under controlled operating conditions to ignore the effects of changes in seagrass distributions, flow, and weather.

2.3 Numerical simulation using simplified bay model

For the numerical simulation, particle tracking calculations are performed using a simplified bay model and a model of Ago bay as an actual sea area. Using these models, sampling points, sampling times, and the unique relationship with eDNA occurrence location are investigated. eDNA with different points of origin do not coexist, a monitoring regime can be established by tracking changes in the amount of eDNA in samples obtained regularly at a fixed point on the predicted route.

2.4 Field survey and eDNA analysis in Ago bay

eDNA spatial distribution surveys were carried out in Ago bay, Mie Prefecture, where the growth of *Z. marina* has been confirmed. *Z. marina* in Ago bay is mainly annual, germinating in winter, growing in spring, and then dying and flowing out during the summer. With this seasonal change in biomass, it was selected as a target area.

3. eDNA ANALYSIS METHOD FOR EXPERIMENT AND FIELD SURVAY

3.1 Seasonal variations in eDNA in the water of a mesocosm experimental tank

The mesocosm experiment with a *Z. marina* bed has been continuing since February 2004 at Port and Airport Research Institute, and the biomass of *Z. marina* changes seasonally like that of natural *Z. marina* beds (Hosokawa et al., 2009). *Z. marina* was grown in the tank under controlled physical conditions such as tides and seawater exchange rates (Figure 1(a); 3m x 2m x water depth 0.5-1.0m, 12.4h cycle). About 24% of the water in the tank is exchanged per tide on weekdays.

1L of surface water was sampled twice a week for nine months from July 2018 to March 2019. The water tank underwent a change in water with the tides; it was drained during the ebb tide and newly pumped seawater flew in during the flood tide. The water samples were collected at low tides because water residence time in the tank was the longest in a tide at this time and sufficient eDNA would have been released just before new seawater flowed in. The heights of five arbitrary leaves were measured in area III shown in Figure 1(a), and the densities were measured at three locations in the observation area ($400cm^2$) installed in area IV shown in Figure 1(a). Any detached seagrass leaf was removed daily from the tank.

3.2 Laboratory experiment of eDNA analysis

A laboratory experiment was conducted to confirm differences in DNA count with particle size and to confirm the degradation characteristics of eDNA. The experiment of particle size is important as morphological information for numerically modeling the eDNA. A 1L sample was filtered in the order of 20, 8, 6, 0.7, and 0.3μm and each filter was analyzed. To determine eDNA degradation characteristics, collected samples were

stored at room temperature in a dark place for up to seven days from the date of collection and analyzed every day.

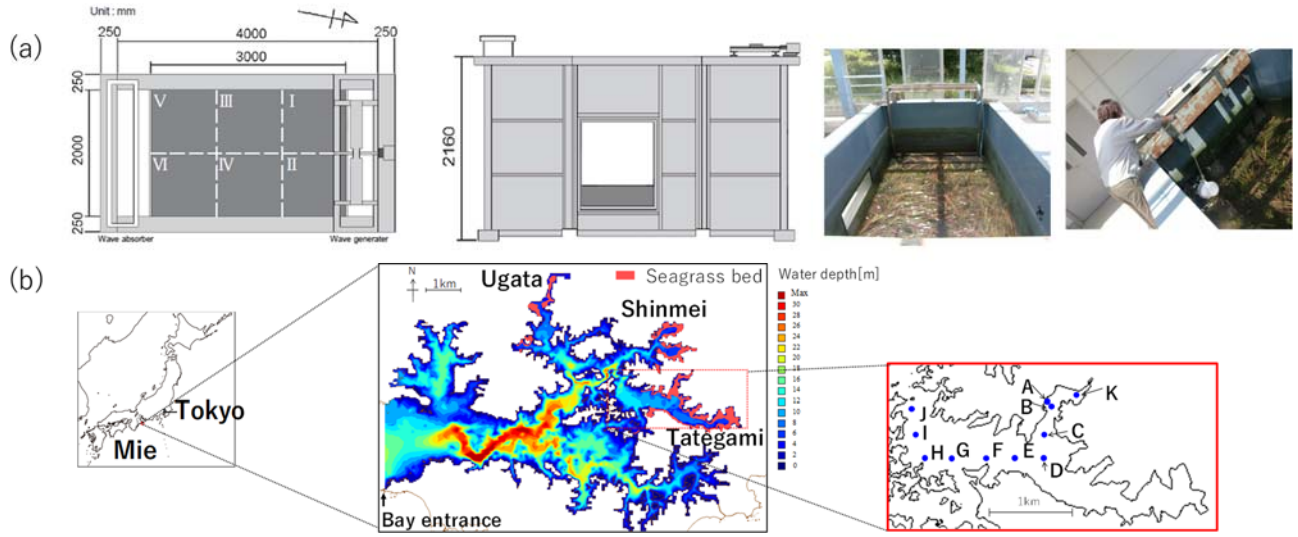

Figure 1. Study field: (a)water tank dimensions and photograph, (b) research area (Ago bay), seabed topography, distribution of seagrass bed and sampling point.

3.3 Field survey in Ago bay

Ago bay is a remarkable Rias coast, a closed bay with large and small islands located in the center of the bay. According to a 2010 survey by Mie Prefecture, *Z. marina* is mainly distributed in shallow areas along the coastline deep in the Tategami, Shinmei, and Ugara bays (Mie Prefecture, 2011). The survey was conducted in Tategami bay, which has been widely known for *Z. marina* growth (Figure 1(b)).
Water sampling and diving surveys in January 2018 and January 2019 at 11 points (A to K) about 3km from the inner bay where *Z. marina* grows to the bay entrance (Figure 1 (b)) was carried out. About one hour after low tide, seawater was sampled from a point 1m below the surface (in a water depth of 0.5-1.5m) with a water sampler from point K at the bay entrance side to point A at the inner bay side. The sampled seawater was dispensed into 1L containers, 1ml of benzalkonium chloride (10 w/v%) was added to suppress DNA degradation (Yamanaka et al., 2016) and the samples were then refrigerated. Visual observations of *Z. marina* coverage and plant height by divers immediately below the sampling point were recorded.

3.4 Analysis method of eDNA

3.4.1 Filtration and extraction

The day after sampling, the 1L sample of seawater was filtered through a glass-fiber filter (0.7μm, Φ47mm), and the filter paper was stored frozen (-20°C). The day before the quantitative polymerase chain reaction (qPCR) analysis in described in section *3.4.2* below, 100μl of a DNA extract was obtained from the filter paper using DNeasy Blood & Tissue kit (QIAGEN).

3.4.2 qPCR analysis

To compare the *Z. marina* DNA amount, qPCR analysis was performed under cycling parameters using the primers (forward:5'-GCTTTCTTTTCCCTTACACGAG-3'; reverse:5'-AACCTTCCCAACCCCGTT-3'; and a TaqMan probe:5'-FAM-TTCATGATTGGTTGATATAT-MGB-NFQ-3'), including an initial denaturation step at 50°C for 2 min and 95°C for 10 min; 55 cycles of 95°C for 15s and 60°C for 1 min. The reaction solution was 10μl of 2 × Environmental Master Mix, 0.1μl of AmpErase, 900nM each of the primers, 125nM of the probe, and 2μl of DNA template to 20μl by sterile distilled water (SDW).

3.4.3 Confirmation of assay inhibition effect of humic acid

Humic acid has been reported as one of the problematic inhibitors in DNA analysis (Mckee et al., 2015), and it is important to confirm its effects due to its presence in seagrass. To confirm the effect of humic acid on PCR inhibition, humic acid (brown forest soil) from the Japan Humic Society was used to confirm the effect of humic acid on analysis of artificial gene amplification. As for the artificial gene, qPCR analysis was carried out using *Z. marina* and λ phage (Honjo et al., 2010) was used for inhibition confirmation. 2μl of an artificial gene (3000 copies), 2μl of a filter paper extract obtained by filtering distilled water, and 200 to 2000ng of humic acid were added as DNA templates to the qPCR reaction solution, and the total volume was adjusted to 20μl with SDW.

4. NUMERICAL SIMULATION METHOD

4.1 Calculation conditions of numerical simulation

In eDNA analysis, a sampling seawater is filtered and the filtration residual matter remaining on the filter paper is target of the analysis. The eDNA is an extremely fine solid, not dissolved matter. A particle tracking simulation method was adopted and carried out to forecast routes of eDNA leaving seagrass beds.

The simulation was carried out for two types of bathymetry. They are simple virtual bay and the Ago bay as an actual sea. The simulation process is below. At first, hydrodynamic simulation using tide as an external force using Delft 3D-flow (Delteares, 2014) was done. Flow velocity components were extracted for each element of the calculation grid at 10-minute intervals and flow velocity was linearly interpolated over time. The eDNA was treated as a particulate, and the particles were tracked at 3-second intervals. Table 1 shows the main calculation conditions. The eDNA was assumed to be an extremely fine material consisting of plant fragments released from the seagrass beds and the particles were treated as completely passive in the flow. Particle source areas were seagrass beds A to C (colored areas in Figure 2 (a) and (b)) and specific origin areas, m1 to m10 (red areas in Figure 2 (c)). The seagrass origin areas in Figure 2 (a) and (c) were located a distance apart for easy understanding of the results. The particle inputs shown in Table 1 were evenly distributed over the bottom layer in the areas specified. Particles were input every 10 minutes. Changes with time in the number of particles passing each water sampling point were counted.

Table 1. Main numerical analysis conditions for flow and particle tracking calculation

		Simple bay model	**Ago bay model**
Calculation area	East-West	3 km	10 km
	North-West	3 km	8 km
Plane coordinates (Orthogonal linear coordinate system)	Grid count	5,188	18,997
	Grid width	15m	40m
Vertical coordinate	Layer	5	10
Analysis period	Five days (one day ramp-up period)		January 1, 2018 to January 16, 2018 (ten days ramp-up period)
Boundary condition (open sea)	Tidal	M2	Predicted tidal level at Owase, Mie Prefecture, Japan (Japan Meteorological Agency)
Particle input	Number	1083 per cell	972 per cell
	Time step	10 minutes	

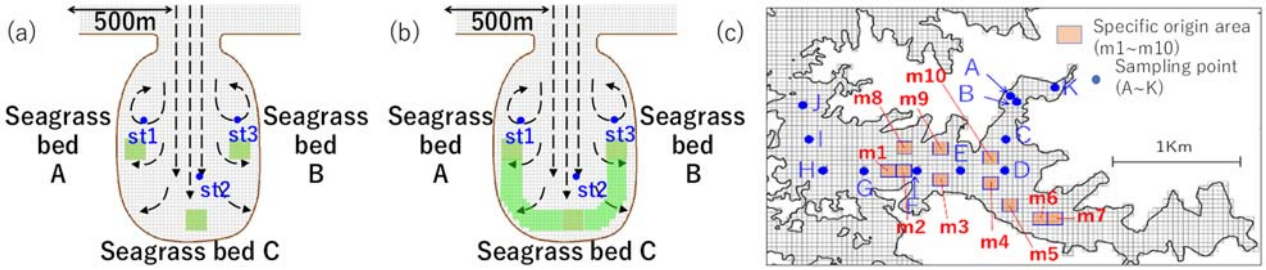

Figure 2. Particle input areas (seagrass beds) in each model. (a) simple bay model-1, (b) simple bay model-2 and (c) Ago bay model.

4.2 Model

Figure 2 shows the shapes of simple bay and Ago bay. The simple bay model has bay mouth of inflow/outflow from/to the open sea. Vectors in figure 2 (ab) show flow patterns at flood tide calculated by the hydrodynamic simulation. Circulation flow has made near the bay mouth. At ebb tide, these circulation flows disappear, and the flow patterns become unidirectional. The bathymetry of simple bay is bowl shape with a maximum depth of 8m in the center of the bay. Water sampling points are at st1, st2, st3 shown in Figure 2. The number of particles (the particle count) passing through each cell (2m×2m×depth of water) was counted. The volume of cell is too large in comparison to actual our 1L of water-sampling method, but we considered that the current model can discuss the time-varying tendency of the number of particles passing through a cell. The reason is we confirmed that the number of passing particles tends to be proportional to the cell volume.

In Ago bay model, specific origin areas (red areas in Figure 2 (c)) were set as the particle input locations. It was confirmed whether eDNA input in each specific area could be distinguished from eDNA starting from other specific areas. The sampling points are A to K (2m x 2m x water depth 0.5m to 1.5m). We defined 10 areas (m1 to m10) as specific origin areas and examined the possibility that particles starting from these areas could be collected separately from particles starting from the other areas shown in Figure 2 (c).

5. RESULTS OF EXPERIMENTS

5.1 Water tank experiment

Figure 3 shows the results of monitoring *Z. marina* growth over 9-months in the experimental tank. *Z. marina* withered and dropped off between July and October, with height and density both decreasing. From October to November, seagrass height and density were the lowest in the experimental period, and only a few perennial *Z. marina* plants (height 5 to 15 cm) grew. From November, germination from rhizomes began to be observed in parts of the aquarium outside the observation zone (areas I and II in Figure 1(a)). By the end of March, density increased because of the germination period and plant height increased.

The eDNA particle count (Figure 4 (a)) were high in July, then temporarily decreased before increasing again until mid-August. The eDNA copies decreased from mid-August to October and reached a low value of several hundred copies/L from October to November. From December, when germination and growth began to be observed in the aquarium, the eDNA copies were 1000 to 2000 copies/L, and thereafter the amount of eDNA tended to increase. The period of decreasing eDNA copies coincided with that of the outflow of the *Z. marina* after August, with the biomass in the water tank decreasing. From December, when winter germination and growth progressed, the amount of eDNA gradually increased, albeit slowly.

The relationship between biomass and eDNA copies was approximately linear for both height and density (Figures 4 (b), (c)). This result suggests that eDNA copies would be a suitable measure for examining the temporal changes (increase/decrease) of seagrass biomass. Although the cause of the temporal decrease in eDNA in July is unclear, seagrass runoff was small during this early stage of withering, whereas seawater exchange in the aquarium was irregular before and after the sampling period. Interference in the analysis due to an increase in humic substances may also be considered.

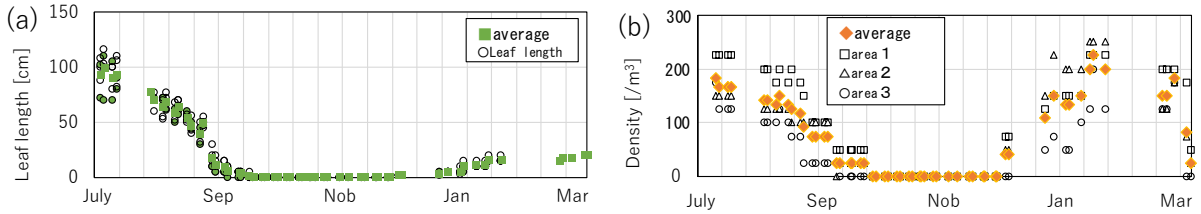

Figure 3. Monitored *Zostera marina* growth: (a) plant height and (b) plant density

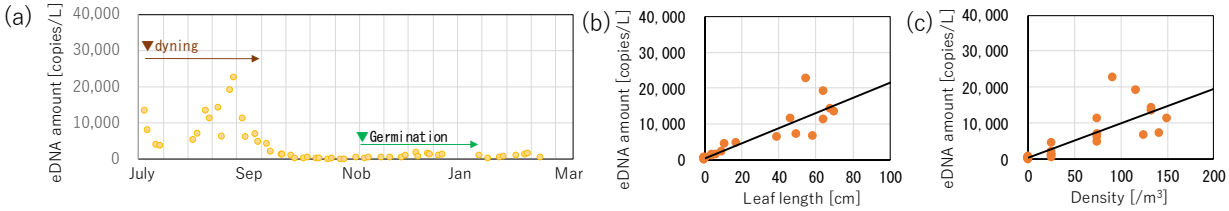

Figure 4. Results of eDNA analysis: relationship between eDNA (from August to October) and plant height (b) and plant density (c).

5.2 Laboratory experiments

In the particle size study, no DNA was detected from the 20, 8, and 6μm filters, but detected from the filters of 0.7μm or less (Figure 5 (a)). It was speculated that, although the objective was eDNA from plant fragments, the DNA extraction method used did not include a cellulose decomposition step making it suitable for chloroplasts (of about 5μm), which is smaller than plant fragments.

Figure 5 (b) shows the test results of eDNA degradation characteristics. The detection ratio is calculated using the amount of DNA on day 0 as the denominator and the amount of DNA on the elapsed day as the numerator. According to Figure 5 (b), the ratio fell by almost half after two days and was down by about 80% after three to seven days.

Figure 5 (c) shows the assay results for inhibition by humic acid. The Ct value on the vertical axis is the number of qPCR cycles required to amplify the DNA in the qPCR solution to a predetermined DNA concentration. The smaller the Ct value, the more DNA was contained in the PCR solution. An increase in the Ct value due to the addition of humic acid indicates that the qPCR was inhibited, and the inhibition is calculated as a DNA concentration that is lower than the original value. In Figure 5 (c), the higher the humic acid concentration, the higher the Ct value and the greater the inhibitory effect. qPCR analysis was also performed by adding artificial seagrass genes to extract liquid from a filter paper of the seawater in the tank (December). The Ct value obtained was ±0.1, it means a small variation, and it confirms that there was no obstruction in the seawater of the tank in December. In the future, similar confirmation will be performed using samples with a large amount of DNA,

and the factors that greatly reduce the amount of DNA during the period of high biomass, as seen in July in Figure 4 (a).

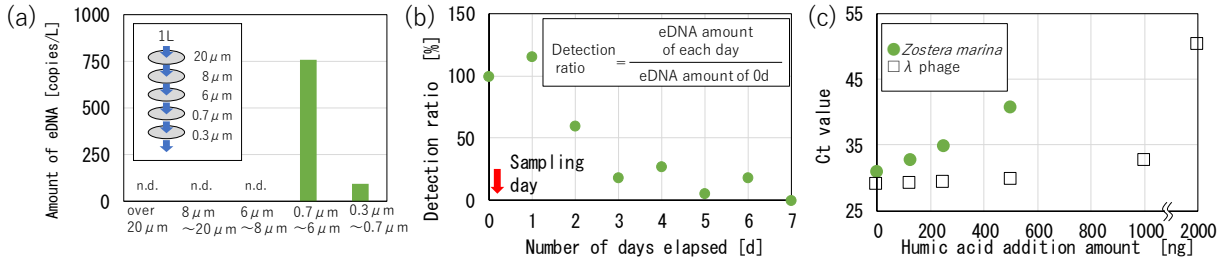

Figure 5. Results of laboratory experiments. (a) the particle size of eDNA, (b) detection ratio of eDNA and (c) qPCR results for inhibition by humic acid

6. RESULTS OF NUMERICAL SIMULATION

6.1 Simple bay model

The velocity vectors and the distribution of particles generated from the seagrass beds (A, B, C) are shown Figure 6. Figure 7 plots the changes over time in the number of eDNA particles at the sampling points (st1, st3). In the simple model, the flow was unidirectional toward the bay entrance during the falling tide, and a circulating flow was formed in the bay during rising tide. Particles in the initial distribution (Figure 2 (a)) generated by each seagrass field move toward the bay mouth at the time of low tide (Figure 6 (d): showing the time of the first low tide after the particles are injected). The particles then moved to the left or right due to the circulating flow at the time of the rising tide (Figure 6 (e): showing the first rising tide after the particles were injected). Repeated movement of particles located near the bay mouth and at the center of the bay, driven by the circulating flow generated during the rising tide, causes the blue particles (starting from seagrass bed A) to move into the vicinity of st3 through the center of the bay. They mixed with green particles that started from seagrass bed B. The time distributions shown in Figures 6 (d) and (f) confirm that, at low tide, only particles from the nearest seagrass beds A and B arrive at st1 and st3, respectively. In this situation, monitoring is considered possible. On the other hand, at high tide (Figure 6 (g)), particles did not pass through st1 and blue particles passed through st2. At st3, green and blue particles were mixed and were indistinguishable. It means the monitoring in st1 and st3 is considered difficult. During the rising tide, between low tide and high tide, blue particles originating from distant seagrass ground A appear exclusively at st3 (Figure 7 (b)). This makes it possible to monitor a remote seagrass ground. Since the particles originating from seagrass beds A and B may appear at st3 at different times, there is a possibility that two seagrass beds can be monitored separately by appropriate sampling

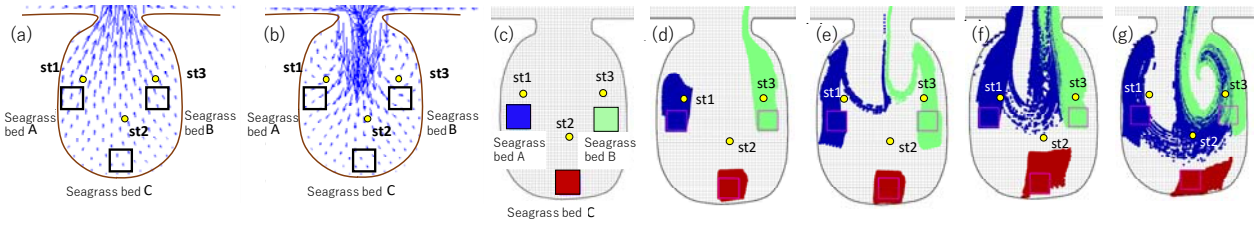

Figure 6. Flow velocity vector ((a), (b)) and particle distribution of simple bay model((c) ~ (g)). (a) Maximum of ebb tide, (b) Maximum of flood tide, (c) initial position, (d) first time of low tide, (e) first time of high tide (f) low tide and (g) high tide.

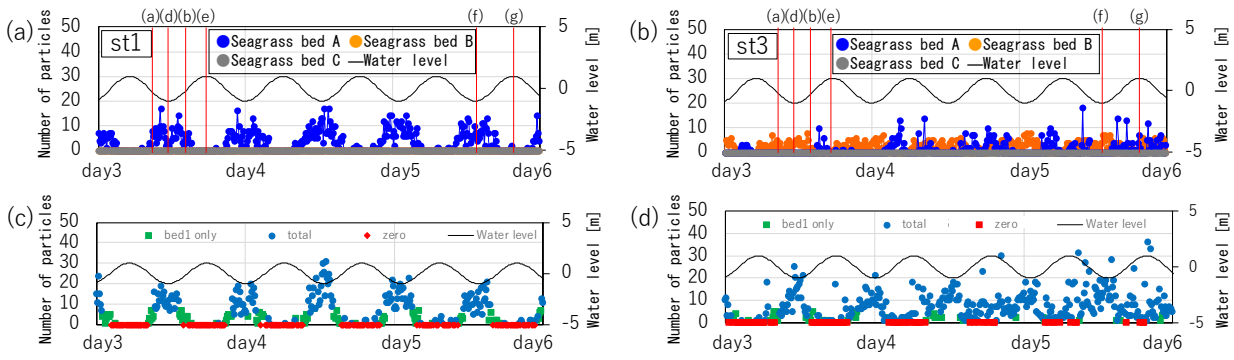

Figure 7. Number of particles leaving each seagrass bed. (a) st1 and (b) st3 by simple bay model-1, (c) st1 and (d) st3 by simple bay model-2.

water at one point. Figure 6 also shows that there are points in the bay through which no particles passes due to circulating flow, so the selection of sampling points and timing is important. The calculation with continuous seagrass beds (Figure 2(b)) resulted in a time for capturing particles starting from multiple locations (blue plots) and a different time for capturing particles starting only from seagrass beds A and C (green plots) (Figure 7 (c), (d)). This suggests that, even in a continuous seagrass bed, depending on the flow and sampling points, particles starting from a specific area may be monitored.

6.2 Ago bay model

The change over time in the number of particles starting from specific areas (m1 to m10) is shown for sampling points D and E (Figure 8). Sampling points were defined in the range of 2m x 2m x water depth of 0.5m to 1.5m to simulate actual sampling conditions. There was a tendency to increase or decrease over time. At point D, many particles appeared and the most frequently occurring particles are those starting from m3 and m10. At around January 15th (12:00), particles from m10 appeared exclusively for about 3 hours. At point E, many particles starting from m10 appeared at January 16th (0:00). Particles that started from m10 appeared at low tide at points D and E. Particles starting from m10 were transported deep into the bay, to points D and E, during rising tide. It can be inferred that point E has been reached. From the above, the number of passing particles is several, and it is considered that there are not enough particles to declare, however, it is possible that the appearance of particles starting from a particular area may be indicated by the flow of actual sea area. The situation is more complicated than with the simple bay model; particles may appear intermittently, may appear more prominently than others depending on the specific location, or may appear exclusively at certain times. This means that even if DNA is generated from multiple seagrass beds at the same time, there are moments when DNA originating from a specific bed passes a specific point exclusively. Water is collected at these points and times it is thought that distinguishable DNA can be collected. That is, it was suggested that this method might be applicable depending on the combination of eDNA distributions and flows.

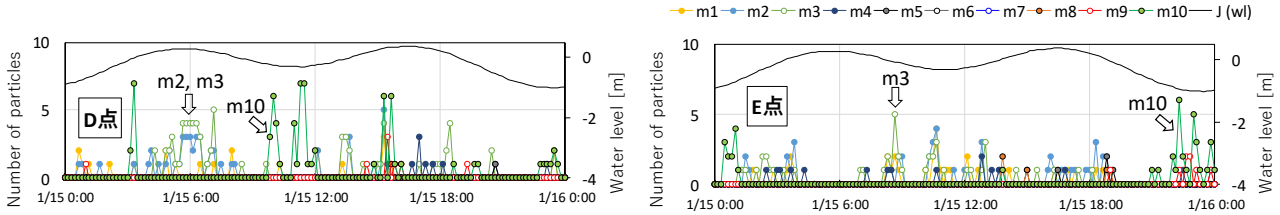

Figure 8. Temporal change of particle numbers in Ago bay model on January 15th. (a) point D and (b) point E.

7. SURVEY AND eDNA ANALYSIS RESULTS IN AGO BAY

7.1 Results of survey

As shown in Figure 9, in January 2018, the coverage was 5% to 40% from points K to G in the inner part of the bay, and *Z. marina* was flourishing widely. In January 2019, a coverage of 30% was confirmed near point E, but at other points only a little *Z. marina* was confirmed to be present (less than 5% coverage). These field surveys were conducted in the same germination period in January of each year, but *Z. marina* growth conditions (cover, plant height) and growth area differed greatly (Figure 9 (c)). This suggests that high water temperatures may have delayed germination in 2019. The germination delay in 2019 was also confirmed in the water tank.

7.2 Results of eDNA analysis

Water was sampled from 11 points (A to K). Figure 9 (a) and (b) show the results of the qPCR analysis. The eDNA counts were several hundreds copies/L, except at point F in 2018, which was around the lower limit of quantification, resulting in a highly variable analysis result. Since the amount of environmental DNA is lower than the amount in the aquarium experiment, the amount of sampled water is considered to be insufficient. Although there is necessity to improve quantitative comparison, such as increasing the amount of water sampled,

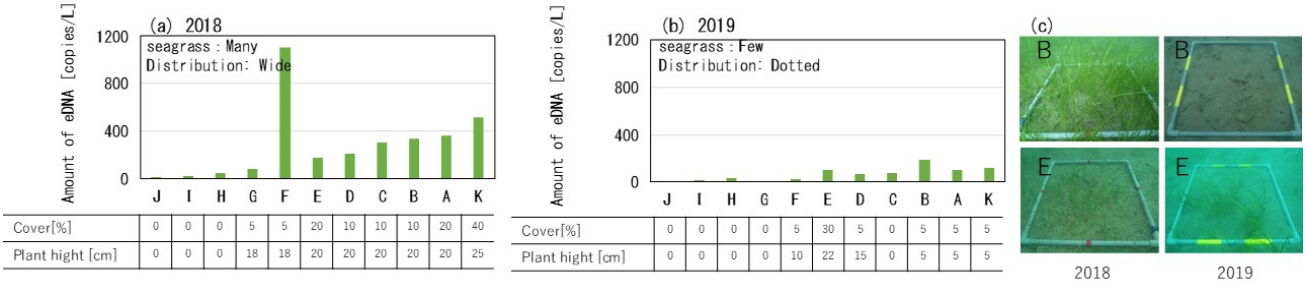

Figure 9. eDNA analysis and survey results in Ago bay. (a)2018, (b) 2019 and (c) growth comparison of seagrass at points B and E.

it was decided to use these results to verify detection trends at multiple points in the actual bay. According to the 2018 and 2019 survey results, *Z. marina* had a high concentration in the inner bay, and the eDNA amount tended to be low at points where there was no visual confirmation of seagrass below the surface (points H to J). In 2019, when growth was slower than in 2018, the overall DNA counts were low.

8. CONCLUSIONS

In this study, a seagrass monitoring method was aimed to establish from two perspectives: eDNA characteristics of nine months with changes in *Z. marina* and a particle tracking calculation simulating eDNA using a simple bay model and a model of Ago bay. In numerical analysis, the simple bay model shows that eDNA particles originating from a specific seagrass bed may be dominant although the timing at which this occurs varies by the location or flow. In addition, it is shown that particles departing from a specific bed sometimes appear to the exclusion of particles departing from other beds in the Ago bay model. This suggests that even if DNA is generated simultaneously from multiple seagrass beds, there are certain point of opportunity during which DNA originating only from a particular bed is passing a particular point, and that DNA distinguishable from other starting points can be collected by collecting water at during those point. It is confirmed that it might be possible to establish a method based on these findings depending on the particular seagrass distributions and flows. In water tank and laboratory experiment, it was suggested that the eDNA amount of seagrass was related to the seasonal change in biomass. In the field survey, although the seagrass distribution was not fully understood because it was a limited survey by divers near the sampling point and eDNA analysis also has some issues, such as water sampling amount, the eDNA tended to be high at times when the amount of seagrass was high. These results suggest the possibility that changes in biomass can be captured by using numerical analysis to select water collection points and timings for seagrass field monitoring using eDNA.

In the future, it is important to understand the effects of eDNA analysis inhibition by substances coexisting in seawater and the impact of eDNA fluctuation characteristics in actual sea areas. Also, for quantitative comparison, it is important to increase the amount of DNA to be analyzed, such as by increasing the amount of water collected, to improve accuracy. In the numerical analysis, characteristics of eDNA such as decomposition etc., will be modeled improve accuracy. The relationship between seagrass beds and sampling points will be clarified. This study will be continued to establish a monitoring method based on both eDNA analysis and numerical analysis.

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