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Development of Quantitative Real-Time PCR for Detecting Environmental DNA Derived from Marine Macrophytes and Its Application to a Field Survey in Hiroshima Bay, Japan

Masami Hamaguchi [1,*], Toshihiro Miyajima [2], Hiromori Shimabukuro [1] and Masakazu Hori [3]

[1] Fisheries Technology Institute, Japan Fisheries Research and Education Agency, 2-17-5 Maruishi, Hatsukaichi 739-0452, Japan; bukuro@affrc.go.jp
[2] Atmosphere and Ocean Research Institute, The University of Tokyo, Kashiwa 277-8564, Japan; miyajima@aori.u-tokyo.ac.jp
[3] Fisheries Resource Institute, Japan Fisheries Research and Education Agency, 2-12-4 Fukuura, Yokohama 236-8648, Japan; mhori@affrc.go.jp
* Correspondence: masami@affrc.go.jp; Tel.: +81-829-55-3463

Abstract: The sequestration and storage of carbon dioxide by marine macrophytes is called blue carbon; this ecosystem function of coastal marine ecosystems constitutes an important countermeasure to global climate change. The contribution of marine macrophytes to blue carbon requires a detailed examination of the organic carbon stock released by these macrophytes. Here, we introduce a quantitative real-time polymerase chain reaction (qPCR)-based environmental DNA (eDNA) system for the species-specific detection of marine macrophytes. and report its application in a field survey in Hiroshima Bay, Japan. A method of qPCR-based quantification was developed for mangrove, seagrass, *Phaeophyceae*, *Rhodophyta* and *Chlorophyta* species, or species-complex, collected from the Japanese coast to investigate their dynamics after they wither and die in the marine environment. A trial of the designed qPCR system was conducted using sediment samples from Hiroshima Bay. *Ulva* spp. were abundant in coastal areas of the bay, yet their eDNA in the sediments was scarce. In contrast, *Zostera marina* and the *Sargassum* subgenus *Bactrophycus* spp. were found at various sites in the bay, and high amounts of their eDNA were detected in the sediments. These results suggest that the fate of macrophyte-derived organic carbon after death varies among species.

Keywords: blue carbon stock; marine macrophytes; environmental DNA; quantitative real-time PCR

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1. Introduction

Carbon sequestration and long-term storage in marine systems, including mangroves, salt marshes, seaweed beds and seagrass meadows, is a form of "blue carbon" and constitutes an important coastal ecosystem service for global climate-change mitigation [1–3]. The value of blue carbon ecosystems for greenhouse gas sequestration has recently been calculated to create targeted policies [4], and there is increasing expectation worldwide for blue carbon as a climate-change countermeasure.

Therefore, it is necessary to develop tools that can verify the fate of macrophyte-derived organic carbon, not only in mangrove, salt marsh and seagrass systems, but also the pathway in seaweed habitats along coastlines, which recent research has proven to be another important carbon sink [5–10]. However, research on blue carbon science is relatively new in Japan, and details of carbon sequestration by macrophytes in the coastal environment are still not well understood.

Environmental DNA (eDNA) technology [11–14] is especially useful for studying the contribution of macrophytes to the blue carbon sink. Two eDNA techniques are presently used to investigate organic carbon in marine sediments: comparative metabarcoding using

next-generation sequencing (NGS), and species-specific analysis using quantitative real-time PCR (qPCR). A few studies to date have directly compared these methods of eDNA detection, describing the advantages and disadvantages of each method [15–17].

Compared with conventional methods, metabarcoding with NGS can provide a large amount of genetic information in a shorter time. Therefore, metabarcoding analysis is a powerful tool for determining, for example, the presence and species composition of seaweed eDNA in marine sediments. For this reason, many researchers in the field of blue carbon science have used this method [7,18,19]. PCR primers to amplify the V7 and V9 regions of nuclear 18S rRNA [7,20], or the rbcL gene of plastid DNA [18], are currently being used in NGS. However, these primers can be inefficient because they amplify the DNA of many organisms other than seaweeds, and it is difficult to distinguish between closely related species. Nevertheless, NGS is effective as a primary screening method for detecting the types of macrophytes in eDNA material from marine sediments. Although metabarcoding through NGS originally had the disadvantage of not being quantitative, Ushio et al. [21] succeeded in adjusting the technique to provide quantitative data, thus greatly advancing eDNA technology [22].

TaqMan probes [23], which are hydrolysis probes, or derived dual-labeled probes (DLPs), can incorporate species-specific regions in up to three locations in the primer and probe regions and have extremely high species specificity compared with ordinary PCR. For example, ever faster and more accurate methods are desirable for diagnosing infectious diseases in humans; hence, qPCR using either TaqMan or DLP is frequently used for the diagnosis of SARS-CoV-2 infection, the cause of the current pandemic [24].

The qPCR method is useful for studying the dynamics of specific species in the marine environment because it is less expensive and simpler than NGS. In qPCR, using either TaqMan or DLP, up to 5 or 6 targets can be examined simultaneously, although the quantitative detection of 2 or 3 targets simultaneously is generally used [25]. Taking advantage of this property of qPCR, Nakayama and Hamaguchi [26] reported a method for the more accurate detection of a single target by simultaneously detecting two different gene regions in the target species, to improve identification accuracy. Hamaguchi et al. [27] designed PCR primers for nuclear DNA, and DLPs for chloroplast DNA, to investigate differences in the persistence of nuclear and chloroplast DNA, respectively, in an effort to detail the dynamics of organic carbon derived from the eelgrass *Zostera marina*, in both the water column and in sediment. If qPCR is applied to detect multiple macrophytes species, it is necessary to design a detection system consisting of DLPs and PCR primers for each target species. This is one of the reasons that there are few examples of the adaptation of qPCR in this field.

It is noteworthy that qPCR is easy to use and inexpensive compared to metabarcoding analysis by NGS, yet it is highly quantitative. Because gene quantification is necessary for an analysis of the relationship between genes and organic carbon, qPCR is useful in studying the degradation process and distribution in the ocean of organic carbon that is derived from species-specific macrophytes. In this work, we present a qPCR-based assay to detect various marine macrophyte species in eDNA. Second, we applied this qPCR system to sediment samples collected in Hiroshima Bay, Japan because blue carbon storage involves sequestration in sediments (for details of the process in wetlands, see [28]). In addition, we verified the newly devised method for evaluating the quantification limits of qPCR, an aspect that has seldom been considered in previous evaluations of eDNA.

2. Materials and Methods

The following qPCR experiments were performed following the guidelines of Busin et al. [29] and the checklist of Raymaekers et al. [30], where possible.

2.1. Sample Collection and DNA Extraction

The Japanese archipelago is surrounded by the Pacific Ocean, the East China Sea, the Japan Sea, and the Sea of Okhotsk, and is connected to the mainland of Asia by the relatively

shallow-lying continental shelf. Although Japan has a small land area, it has a large oceanic area, and there has been much interest in evaluating the ocean as a sink for carbon dioxide. As the islands of Japan stretch from the subarctic to the subtropics, a wide variety of coastal macrophytes occur. A total of 142 species of marine macrophytes, submerged plants, and terrestrial plants were collected in the coastal waters of Japan after 2003. Each sample was dried with silica gel and then crushed with a TissueLyser (Qiagen, Valencia, CA, USA); DNA was extracted with the DNeasy Plant Mini Kit (Qiagen). Sediments from Hiroshima Bay were collected with a Smith–McIntire grab sampler on 29 October 2020 (Figure 1). Hiroshima Bay is located in the western part of the Seto Inland Sea (the largest inland sea area in Japan) and has brackish water and high river inflow. Hiroshima Bay has the most intensive oyster farming industry in the country. The city of Hiroshima is situated on the northernmost shore of the bay, although much of the city area was created by land reclamation and was once a vast expanse of tidal flats and eelgrass beds. Figure 2 shows the locations where different seagrass and seaweed taxa were confirmed in a biomonitoring survey by the Ministry of the Environment of Japan and by the authors' own survey. *Zostera marina*, the *Sargassum* subgenus *Bactrophycus* spp., and *Ulva* spp. were widely distributed in the bay; *Eisenia/Ecklonia* spp. were abundant in the southern part of the bay and around Yashirojima Island; the *Sargassum* subgenus clade *Sargassum* spp. were found only off the northern shore of Yashirojima Island. A fixed amount (~100 mg) of lyophilized sediment was crushed with a TissueLyser, and DNA was extracted with the NucleoSpin Soil kit using the buffer SL-1 (MACHEREY-NAGEL, Deer Park, NY, USA). DNA hypoabsorbent plastic tubes and pipette tips were used in the experiments.

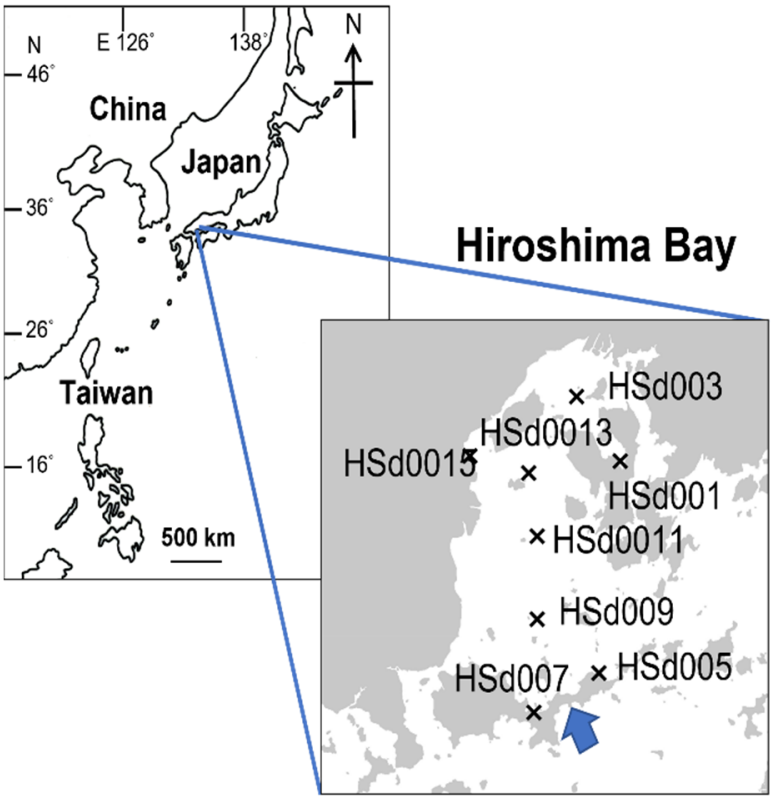


Figure 1. Sampling sites in Hiroshima Bay, Japan.

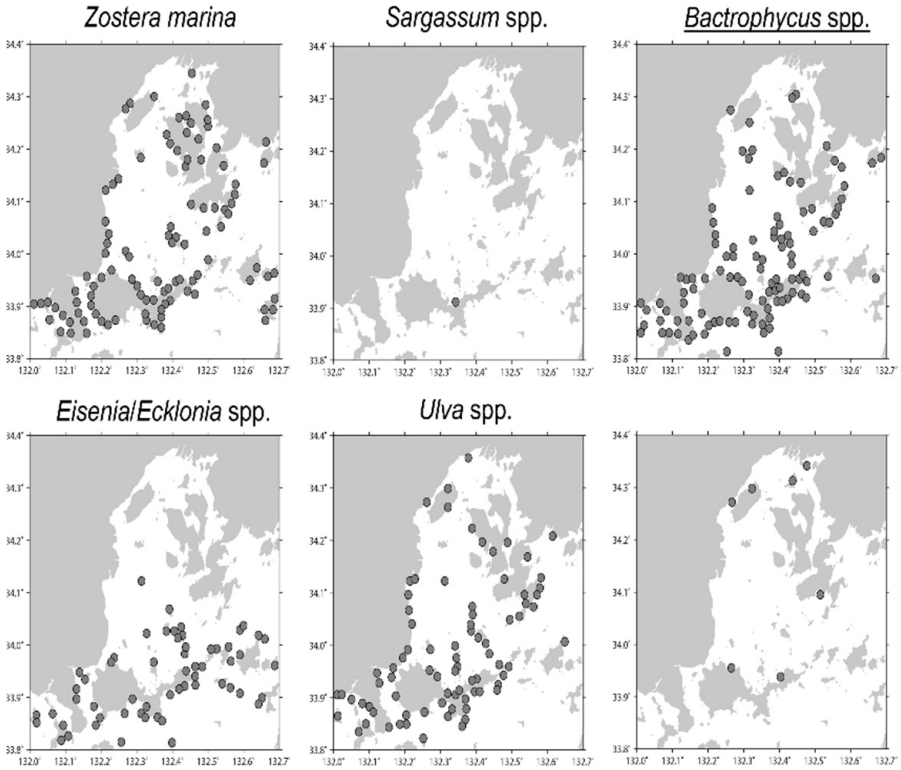


Figure 2. Distribution of various macrophyte taxa in Hiroshima Bay. The solid circles show each species' observed site.

2.2. Quantitation of Extracted DNA and Plasmid DNA

Extracted DNA and plasmid DNA were quantified and qualified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) or GeneQuant II (Amersham Pharmacia, Uppsala, Sweden) spectrophotometer. The closed circles show the site at which each species was observed.

2.3. DNA Sequences of Internal Transcribed Spacer (ITS) and Maturasek (matK) Genes of Various Macrophyte DNA

Following the method of Hamaguchi et al. [27], the ITS gene of nuclear DNA and the matK gene of chloroplast DNA were subjected to polymerase chain reaction (PCR) amplification using primers that were reported previously for ITS [31,32] and matK [33]. A MyCycler™ thermal cycler (Bio-Rad, Hercules, CA, USA) was used to amplify PCR products in a total volume of 25 μL, comprising 1 U of KOD FX™ (1 U/μL; ToYoBo, Osaka, Japan), 2 × PCR buffer for KOD FX, 2 μM of each dNTP, 0.3 μM of each primer, and 1 μL (DNA concentration < 100 ng) of template DNA. This DNA polymerase is resistant to contamination by plant-derived polysaccharides and other substances that inhibit PCR, making it suitable for PCR using DNA extracted from various seagrasses, macroalgae, and mangrove species. The PCR amplification cycles included denaturation at 94 °C for 2 min; 35–45 cycles of denaturation at 94 °C for 10 s followed by annealing at 55 °C for 30 s and an extension at 68 °C for 60 s; and a final extension for 5 min at 68 °C. The PCR-amplified products were checked by loading 3 μL of each sample with 3 μL of loading dye on a 2% agarose gel (Agarose S; Nippon Gene, Tokyo, Japan) containing 0.5 μg/mL of ethidium bromide. The remaining 22 μL of PCR product was subsequently purified using a QIAquick PCR Purification Kit (Qiagen). If the PCR product was heteroplasmic, then it was subcloned using a Zero Blunt TOPO PCR Cloning Kit for sequencing (Thermo Fisher Scientific), according to the manufacturer's protocol. Positive clones were checked by colony-directed PCR, then by electrophoresis, and the PCR products were purified using the above methods.

The purified PCR amplicons were sequenced using the PCR primers described above and the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Waltham, CA, USA) in a 3130xl Genetic Analyzer automated DNA sequencer (Applied Biosystems).

2.4. Design of Primers and DLPs for Detecting Various Macrophyte DNA

There are generally two types of qPCR detection systems: the intercalation method and the hydrolysis probe method (e.g., TaqMan probes). Although the former is easy to design, it has low specificity, and the operation is complicated because the obtained PCR product needs to be verified. Therefore, in this study, the hydrolysis probe method was adopted as the system for qPCR. The DLPs and PCR primers for detecting macrophyte DNA of various taxa were designed using the sequence data of individual specimens that we collected, and the sequences are registered in the international DNA database. Because a portion of the macrophytes species is classified according to country, we used the sequence information deposited by Japanese researchers as much as possible among the nucleotide sequences registered in the international DNA database. DLPs and PCR primers were designed for all macrophytes in the ITS region, and for angiosperms such as mangroves and eelgrasses in the matK region. Stiger et al. [34] divided the genus *Sargassum* into two subgenera, *Sargassum* and *Bactrophycus*, based on the ITS region. Although there are exceptions, based on the data presented here, members of *Sargassum* are likewise divided into these two subgenera because the subgenus *Sargassum* contains many subtropical species and the subgenus *Bactrophycus* includes many temperate species. To discriminate between *Saccharina japonica* and *S. longipedalis* (*Phaeophyceae*), DLPs and PCR primers were designed in the cytochrome c oxidase subunit I (COI) region of mitochondrial DNA, in addition to the ITS region, in accordance with the method used by Yotukura et al. [35] and Rana et al. [36].

Extrapolative internal standards used to determine the inhibitory effect of impurities and matrix effects remaining in the sample during field sample analysis on the PCR reaction were prepared from two species: for the internal standard, we used DNA from *Crassostrea zhanjiangensis*, a subtropical oyster species newly described in 2013 [37] but not yet confirmed in Japan, and from *Ammodytes hexapterus* [38], a subarctic fish found north of Hokkaido. DLPs and PCR primers were designed on the partial sequence of COI or cytochrome b (CYTB) of the mitochondrial DNA of these species, respectively. The designed DLPs and PCR primers were tested for specificity in silico using the international DNA database, and the specificity was also checked using samples that we collected that were closely related to each target species. First, qPCR, using the designed DLPs and PCR primers, was performed via the temperature-gradient function of the real-time PCR instrument, to investigate the appropriate reaction temperature and its range. Next, qPCR was performed with the designed DLPs or PCR primers, using the DNA of each land plant and marine macrophyte species collected from various locations to verify the species specificity. Species specificity was judged to be present if the difference in quantification cycle (Cq) values between the species for which the DLP or PCR primer was designed and those of the species to be compared was 10 or more. When designing DLPs and PCR primers, we followed the work of Bustin et al. [29] and Pedersen et al. [12] as closely as possible.

2.5. Plasmid Development for Construction of the Standard Curve

The PCR amplicons of newly designed PCR primers included the target region of the DLPs and the PCR primer sets designed in this study. Each amplicon was cloned into a pCR4-TOPO plasmid vector (Thermo Fisher Scientific) and chemically transformed into *Escherichia coli* TOP10-competent cells (Invitrogen), cultured overnight on Luria–Bertani (LB) agar plates (Thermo Fisher Scientific). After this period, several colonies were selected and checked via direct colony PCR using the primers for each of the inserted PCR amplicons. Six positive colonies were further cultured in 2 × LB broth overnight, and plasmids were

extracted using a QIAprep plasmid purification kit (Qiagen). The construction of the calibration curve followed the method of Hamaguchi et al. [27] and used a DNA polymerase selected according to the characteristics of the DLPs and PCR primers designed in this study, making a 10-fold dilution series of 10^1 to 10^7 using six plasmids. The calibration curve was redesigned if the obtained amplification efficiency was out of the range of 0.85–1.100, if the slope was out of the range of −3.6–3.1, or if the coefficient of determination of the calibration curve was <0.98. These criteria were based on the methods of Broeders et al. [39] and Ma et al. [40] but were set to more stringent conditions.

2.6. Application of the Newly Developed qPCR System to Field Sediment Samples

DNA was extracted from samples of marine sediment in Hiroshima Bay collected by the research vessel Shirafuji-Maru on 29 October 2020 (Figure 1) using the method described above and was then analyzed to identify macrophytes in the eDNA. The eDNA of seaweeds and seagrasses was analyzed by targeting *Zostera marina*, *Sargassum* spp., *Bactrophycus* spp., *Ulva* spp., *Neopyropia yezoensis* and its species complex, and *Ecklonia/Eisenia* spp. A duplex qPCR and well-to-well correction were performed in a final volume of 10 µL, containing 5 µL of SsoFast Probes Supermix with ROX or iQ Multiplex Powermix (Bio-Rad), 0.3 µL of each primer (300nM final concentration), 0.2 µL of each DLP (200nM final concentration), 1 µL of template DNA, and 3.5 µL of sterilized pure water. Amplification reactions were carried out using the CFX96 Touch Real-Time PCR detection system (Bio-Rad). For thermal cycling conditions, when the DNA polymerase used was SsoFast Probes Supermix with ROX, the reaction was carried out for 2 min at 95 °C, followed by 59 cycles of 10 s at 95 °C and 20 s at the reaction temperature of the respective DLP or PCR primer. On the other hand, when the DNA polymerase was iQ Multiplex Powermix, the reaction time was 2 min at 95 °C, followed by 10 s at 95 °C, and 20 s at the reaction temperature of the respective DLP or PCR primer for 59 cycles. Prior to the analysis, DNA samples extracted from the sediment samples were spiked with an internal standard made from *Crassostrea zhanjiangensis* or *Ammodytes hexapterus* DNA, and qPCR was performed to determine the presence of inhibitors of the PCR reaction. The thermal cycling conditions were 59 cycles of 95 °C for 2 min, followed by 10 s at 95 °C and 20 s at 62 °C using the SsoFast Probes Supermix with ROX. As a result of the analysis, if the detected amount of the internal standard shifted by more than one order of magnitude, it was judged that the PCR inhibitor had an effect, and the following operations were performed. The effects of PCR inhibitors and the matrix effect were reviewed by Schrader et al. [41] and Sidestedt et al. [42]; if their removal was necessary, a PCR inhibitor removal kit (Zymo Research, Irvine, CA, USA) was used or else DNA samples were diluted with tris-buffered (10 mM, pH 7.5) sterile water.

2.7. Calculation of Limit of Detection (LOD) and Limit of Quantification (LOQ) for eDNA

To standardize the eDNA data, the values of LOD and LOQ were determined using the number of copies of eDNA in sediments from Hiroshima Bay as a preliminary analysis. Then, six samples were selected whose DNA copy number was $\leq 10^3$, and each sample was analyzed in eight replicates. First, the DNA copy numbers obtained from these results were converted to normal logarithms. Then, the mean standard deviation (δ), and coefficient of variation (CV) were calculated from these values. Finally, we selected the sample with the lowest DNA copy number with a CV of <10% and calculated the limit of detection as 3.3 δ and the limit of quantitation as 10 δ, according to the guidance document of the European Union Reference Laboratories [43].

2.8. Analysis of Physical and Chemical Properties of the Sediment Samples Collected from Hiroshima Bay

After the initial processing of the sediment samples, total nitrogen, organic carbon, C/N ratio, nitrogen and carbon stable isotope ration, specific surface area, total pore volume and average pore diameter were determined, according to the methods of Miyajima et al. [44,45]. Statistical analyses, using Type I regression, were performed in R 3.1.0 [46]

within SPSS 22.0 (IBM, Armonk, NY, USA); *p*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Design of Primers and DLPs for Detecting DNA of Various Macrophyte Taxa

Eight species of mangrove, 14 species and 2 local populations of seagrasses, 47 species of *Phaeophyceae*, 22 species of *Chlorophyta*, 19 species and 1 culture strain of *Rhodophyta*, 2 species of calcareous algae, 6 species of freshwater submerged plant, and 23 species of terrestrial plants from Japan were collected to design the DLPs and PCR primers for qPCR (Tables 1–5), and the species specificity of the qPCR system was checked using these samples (Tables 1–6). In Table 1, the species for which DLPs and PCR primers were designed are indicated by the solid circles in the columns referring to Tables 1–5, and species for which the specificity of the designed DLPs and PCR primers was verified are indicated by open circles. The characteristics of the calibration curves are shown in Table 6. The amplification efficiency, slope, and coefficient of determination of the DLPs and PCR primers designed in this study were all within the range of the set criteria. In this study, calibration curves were prepared using a dilution series from 10^1 to 10^7 copies/μL and showed high linearity, with a coefficient of determination of 0.99 to 1 (Table 6). Therefore, the dynamic range is 10^1 to 10^7 copies/μL for all DLPs and PCR primers. However, in this study, the ITS regions of *Rhizophora stylosa*, *Bruguiera gymnorrhiza*, *Undaria pinnatifida*, and *Saccharina japonica* showed different reactivities depending on the DNA polymerase used. When SsoFast Probes Supermix with ROX was used, the amplification efficiency and slope were out of the criteria range, but when iQ Multiplex Powermix was used, these characteristics were all within the appropriate range. For the internal standards for extrapolation, the following DLPs and PCR primers were designed using *Crassostrea zhanjiangensis* (CraZha-P: Cy5-CGCGGAAACTGTATCAGC-CACCT-BHQ3, CraZha-F: CAGCCATGCATTGGTTATAAT, CraZha-R:GGACTCAA-AACCTAAACGCA) and *Ammodytes hexapterus* (KitaIkana-Cytb-P: Cy5-CCGACAA-TTTCATCCCTGCCAACCCGC-BHQ3, KitaIkana-CytB-F: CTTTTCAGCCCTAATCTAC-TGGGG, KitaIkana-CytB-R: TGAT-GTGGGGAGGAGTCACG) DNA, and the plasmids obtained by subcloning the PCR products were used. The former was used as an internal standard to be extrapolated to field samples collected in waters north of Kyushu, and the latter for samples from south of Kyushu, Japan.

Table 1. DLPs and PCR primers for three mangrove species in Japan.

Target Species	Target Gene	Abbreviations for DLPs and PCR Primers	Nucleotide Sequences 5′–3′	Reaction Temperature (°C)	Amplicon Size (bp)
Rhizophora mucronata	ITS	YaeITS-P	Cy5-GCGCTGCGACTCCACGATGAGTACC-BHQ3	64.0	116
		YaeITS-F	TCGCGCCAAGGAAATCAAAGA		
		YaeITS-R	ATATCCGTTGCCGAGAGTCG		
	MatK	YehiruMatK-P	FAM-TCATCAGAAGAGGCGTACACTTTGAAGCCA-BHQ1	64.0	169
		YehiruMatK-F	GTCTTTGCTAATGATTTTCCGTCT		
		YehiruMatK-R	ACCACACATAAAAATGACATTGACC		
Bruguiera gymnorrhiza	ITS	OhiITS-P	HEX-GGCCGCTGCGTCTCCACCATCA-BHQ1	64.0	114
		OhiITS-F	GCGCCAAGGAAATCACAGG		
		OhiITS-R	GATATCCGTTGCCGAGAGTCG		
	MatK	OhiMatK-P	HEX-AAGAACCCGCGCTTCTTCCGTCAAAAA-BHQ1	64.0	142
		OhiMatK-F	CTTGTAAACACAAAAGTCCTGTCC		
		OhiMatK-R	CCTCGATATAACGTTTGTGAAATAGAAG		

Table 1. *Cont.*

Target Species	Target Gene	Abbreviations for DLPs and PCR Primers	Nucleotide Sequences 5′–3′	Reaction Temperature (°C)	Amplicon Size (bp)
Sonneratia alba	ITS	MayaITS-P	FAM-ACCTCGCCTTCTCCAGTGTGACAATGATGA-BHQ1	64.0	101
		MayaITS-F	CGGCACTGGATGCTTCCC		
		MayaITS-R	GTGCGAGAGCCAAGATATCCA		
	MatK	MayaMatK-P	Cy5-CCCGTTAGTAAGTCGACCCGGACCGAT-BHQ3	64.0	124
		MayaMatK-F	TTCCCGTTATTCCTTGATTGGATGC		
		MayaMatK-R	CGCACAAATCGGTCGATAATATCA		

Table 2. DLPs and PCR primers for subtropical seagrass species in Japan.

Target Species	Target Gene	Abbreviations for DLPs and PCR Primers	Nucleotide Sequences 5′–3′	Reaction Temperature (°C)	Amplicon Size (bp)
Enhalus acoroides	ITS	Us-ITS-P	HEX-ACCGTTCGTTTCCACGTCACTCGCTC-BHQ1	64.0	99
		Us-ITS-F	ACCTGCGGAAGGATCATTGTC		
		Us-ITS-R	AGGGTTGTTGTTCGGTGGTC		
	MatK	UmishyoB-P	FAM-ACCCGGATACATCACAAAATTGAGCTTTCG-BHQ1	64.0	98
		UmishyoB-F	AGTCCCAGTTATTCCTCTCATTGA		
		UmishyoB-R	CGGATAAATCCGTCCAAATCCC		
Cymodocea rotundata	ITS	Ba-ITS-P	FAM-CGTTCGTTACGTTGGCTACCACTCTCCCT-BHQ1	64.0	91
		Ba-ITS-F	TGCCCATCTCGGAGTTCGTG		
		Ba-ITS-R	GTGGACTCGCCTGACCTGG		
	MatK	BeniA-P	Cy5-CCAAACTGGCTTACTAATGGGATACCCGGA-BHQ3	64.0	120
		BeniA-F	CCCTCTCATTGGATCTTTATCTAAAGA		
		BeniA-R	CATATACGACCAAACCGATCAATAATA		
Thalassia hemprichii	ITS	Rs-ITS-P	Cy5-CATTCACCACACGTCGGGATGCACC-BHQ3	64.0	108
		Rs-ITS-F	GCCGTCCCTGTGTTCCCTA		
		Rs-ITS-R	TCACGACGCACGAAGCAC		
	MatK	RyukyuS-P	HEX-AACCCGTCCAAATCGCCTTACTAACAGGA-BHQ1	64.0	122
		RyukyuS-F	TTCCTCTCATTGGATCCTTGTCA		
		RyukyuS-R	ACATATCCGACCAAATCGATCAATC		
Halophila ovalis Iriomote-type	ITS	Hal-ITS-P	Cy5-ACGTGAAGCAACGAGTGCGATCCACC-BHQ3	64.0	117
		Hal-ITS-F	TCTCGACGATGTCCTGCCTC		
		Hal-ITS-R	GGGGATACTCAGCTTGGGGA		
Cymodocea serrulata	ITS	Ra-ITS-P	FAM-CACTTGAGCACACCGCTTCCACTCCAC-BHQ1	64.0	93
		Ra-ITS-F	GGCCTTCCGGTTCTCTCTCT		
		Ra-ITS-R	CATCGCATCGCATCCATCGA		
Halodule uninervis	ITS	Umj-ITS-P	HEX-TTGCCATCCACCATCCATGCTGCGTC-BHQ1	64.0	98
		Umj-ITS-F	GGCTGAAGTACGTTGGGCTC		
		Umj-ITS-R	GGCGATCCAAGGGAAGCATC		

Table 3. DLPs and PCR primers for temperate and subarctic seagrass species in Japan.

Target Species	Target Gene	Abbreviations for DLPs and PCR Primers	Nucleotide Sequences 5′–3′	Reaction Temperature (°C)	Amplicon Size (bp)
Zostera marina	ITS	AmaITS-P3 AmaITS-F3 AmaITS-R3	FAM-ACCCACCATGCCATGTACCGAACATGC-BHQ1 TGTAAAGAATCAGAGAATGACCTTC CAGTTTCAGAATGGTAAACATTCTAA	64.0	122
Zostera caulescens	ITS	TachiITS-P3 TachiITS-F31 TachiITS-R3	HEX-CGTTTGCCTTGGCAACAATTGTGCCGTG-BHQ1 CTTAAAGGATGCACAATCAAGT ATCGAATGAATACGTTTCACCA	64.0	129
Phyllospadix iwatensis	ITS	Sugamo-P Sugamo-F Sugamo-R	Cy5-AGCCTGCGTGTCGTGCCGTGTAGCGCAGTGTAG-BHQ3 GTGGATTGTTGCAGACGGTTTGTC ACAACGACAGATGGCGCACTAAG	62.0	103

Table 4. DLPs and PCR primers for *Phaeophyceae* seaweed single species in Japan.

Target Species	Target Gene	Abbreviations for DLPs and PCR Primers	Nucleotide Sequences 5′–3′	Reaction Temperature (°C)	Amplicon Size (bp)
Undaria pinnatifida	ITS	WakameITS-P WakameITS-F WakameITS-R	HEX-TCGCCCAACATCGCGTAAC-BHQ1 GCGTCGGTTTTGTAAA TCGAGGGAATTAACCC	56.0	83
Saccharina japonica	ITS	MakonbuITS-P MakonbuITS-F MakonbuITS-R	FAM-CGAGGCGCCCCTCGCCCAACTTCGCA-BHQ1 TATAAATTGTCTGTGAGGCCGCTTCGT TGATTCGAGGGCCTTTTCACAGGCT	68.0	116
Saccharina longissima	ITS	NagaKoCo1-P NagaKoCo1-F NagaKoCo1-R	HEX-GCTGGGACAGGTTGGACGGTGTACCCACCT-GHQ1 CCTCTTTAATTTTGCTTCTAGCGTCTTCATT CCTAAAATAGAAGCAGCACCCGAGAG	68.0	163
Ecklonia kurome	ITS	KuromeITS-P KuromeITS-F KuromeITS-R	FAM-CGCCTCCCTCGGGTTTTAATTA-BHQ1 CGTTTGTAACCTCATCTTT GCCCAACTTCGCATAA	65.0	101
Sargassum horneri	ITS	AkamokuITS2-P AkamokuITS2-F AkamokuITS2-R	HEX-AGCCTCTAGCAACGCTCCAA-BHQ1 TCGCTATATGCAGGTTTA GACTGCCTACCGTCAA	58.0	106
Sargassum muticum	ITS	TamahaITS2-P TamahaITS2-F TamahaITS2-R	Cy5-TGTCATCAGCGCCGCAAAG-BHQ3 GGTGGGTATTTTTGTACC GGAAGACACGGGTTAA	58.0	122

3.2. Detection of eDNA Derived from Mangroves

Table 1 shows the DLPs and PCR primers designed using the ITS and matK genes of the mangroves that are found around the islands of Iriomote and Ishigaki, and have relatively high biomass. Because the mangrove habitats were often defoliated as a result of physiological functions, such as the discharge of salt derived from seawater, we constructed a qPCR system in the matK gene of plastid DNA, in addition to the ITS gene of nuclear DNA, to track their differentiation during decomposition. The newly designed DLPs and PCR primers shown in Table 1 were validated with DNA extracted from 32 species of angiosperms collected in the Yaeyama Islands (Japan's most-southwestern islands) and in Honshu, and none of them reacted by showing a difference in Cq value of within 10, which was the set criterion for the validation of species specificity. The DLPs and PCR primers for mangrove species and the resultant calibration curves are shown in Tables 1 and 6, respectively. The amplification efficiency, slope, and coefficient of determination were within the range of the criteria set in this study, when DNA polymerase was selected according to each DLP and PCR primer.

Table 5. DLPs and PCR primers for *Phaeophyceae*, *Rhodophyta* and *Chlorophyta* seaweed species complex in Japan.

Target Species Complex	Target Gene	Abbreviations for DLPs and PCR Primers	Nucleotide Sequences 5′–3′	Reaction Temperature (°C)	Amplicon Size (bp)
Sargassum spp.	ITS	SubSar-DLP SubSar-F SubSar-R	FAM-GGTGGACTCAGGGGACGAGCAGG-BHQ1 GKGTTCGATCTCGATCTCAAG CAAAGACAATAGAAGCCTGGACAAT	62.0	151
Bactrophycus spp.	ITS	TemSar-DLP TemSar-F TemSar-R	HEX-CGACCCGTCGTACAACGGATCCTC-BHQ1 TGTGCGGGTGAGTTTGAAG CAAACTCACCCGCGYACAT	62.0	102
Ulva spp.	ITS	Ulva-ITS-P Ulva-ITS-F Ulva-ITS-R	FAM-CGGATATCTTGGCTCTCGCAACGATGAAGAACGC-BHQ1 CTGAAGCAGCTTCGYAMGGGGACAC AATGTGCGTTCAAGATTCGATGACTC	65.0	159
Neopyropia spp.	ITS	Susabi-ITS-P Susabi-ITS-F2 Susabi-ITS-R2	HEX-TGGGCGTTGCCCTCTGGAACGTGCT-BHQ1 TCTGACGTAGAGACAGGTGCCGTC CCGTCAAGCACAATCTGCCTCTTTTGA	65.0	105
Ecklonia/Eisenia spp.	ITS	EckEiseITS-P EckEiseITS-F EckEiseITS-R	FAM-TTCTCGGGGTATAAACGCTCGCCTCCCTCGG-BHQ1 GTCTGAGACGTCGCCGTTTGTAACCTCA CCCCTCGCCCAACTTCGCATAACAAA	66.0	119
Saccharina spp.	ITS	SacchaITS-P SacchaITS-F SacchaITS-R	HEX-GTCGCGGCGGCGGACTTTGAGTGTTCCG-BHQ1 ACTCGCCCCTCTTCTCTCCTGTCTCA GAAGCGAGCGCCGTCAACAACTCTG	66.0	150

3.3. Detection of eDNA Derived from Seagrass

Table 2 shows the DLPs and PCR primers specific to the dominant species of subtropical seagrasses found around Ishigaki and Iriomote islands; these were designed for both the ITS and matK genes, following the method of Hamaguchi et al. [27]. Table 3 shows the DLPs and PCR primers for qPCR of the temperate seagrasses *Zostera marina* and *Z. caulescens*, and the subarctic seagrass *Phyllospadix iwatensis*, which were variously found off Honshu, Tohoku, and Hokkaido. DLPs and PCR primers for the matK gene of *P. iwatensis* are under design. The newly designed DLPs and PCR primers that are shown in Tables 2 and 3 were validated by qPCR, using DNA extracted from 39 and 20 species of angiosperms collected in the Yaeyama Islands and Honshu, respectively. As a result, none of them reacted by showing a difference in Cq value within 10, the set criterion for the verification of species specificity in this study. The DLPs and PCR primers for seagrasses are listed in Tables 2 and 3, and the results of calibration curves are given in Table 6. Regardless of the type of DNA polymerase, the amplification efficiency, slope, and coefficient of determination of these DLPs and PCR primers were within the range of the criteria set in this study.

3.4. Detection of eDNA Derived from Phaeophyceae, Rhodophyta and Chlorophyta

Table 4 shows the DLPs and PCR primers for *Phaeophyceae* seaweed single species found in the coastal waters around Japan. Table 5 shows the DLPs and PCR primers for a major macroalgal species-complex that includes species of the subgenera *Sargassum* and *Bactrophycus*, *Ulva*, *Ecklonia/Eisenia* and *Saccharina* in Japan. The construction of most qPCR systems is often designed specifically for a single species. However, this design for a species-complex or genus is a new attempt. The DLPs and primers shown in Tables 4 and 5 were difficult to design, owing to the presence of several closely related species and groups, such as the subgenus *Sargassum*, *Ulva* spp. and *Saccharina* spp. Therefore, to increase the species or group specificity, the DLPs and PCR primers were made longer and the Tm value was set higher than the recommended conditions for their design. The species specificity of the DLPs and PCR primers shown in Tables 4 and 5 were verified using 59 and 68 species of seaweeds, respectively. As a result, none of them reacted by showing a difference in Cq value within 10—the criterion for the verification of species specificity set in this study. The results of the calibration curves are shown in Table 6; the amplification efficiency, slope,

and coefficient of determination were likewise within the range of the criteria set in this study when DNA polymerase was selected according to each DLP and PCR primer.

Table 6. Characteristics of the calibration curves, created with the DLPs and PCR primers designed in this study.

Group	Target Species or Species Complex	Gene	Sloop	Efficiency	R^2	DNA Polymerase
Mangrove	*Rhizophora mucronata*	ITS	−3.60	0.89	0.99	B
		MatK	−3.41	0.96	0.99	A,B
	Bruguiera gymnorrhiza	ITS	−3.64	0.88	0.99	B
		MatK	−3.06	1.12	0.99	A,B
	Sonneratia alba	ITS	−3.34	0.99	0.99	A,B
		MatK	−3.33	1.00	0.99	A,B
Seagrass (subtropical zone)	*Enhalus acoroides*	ITS	−3.29	1.02	0.99	A,B
		MatK	−3.32	1.00	0.99	A,B
	Cymodocea rotundata	ITS	−3.42	0.96	0.99	A,B
		MatK	−3.45	0.95	1.00	A,B
	Thalassia hemprichii	ITS	−3.28	1.02	0.99	A,B
		MatK	−3.24	1.04	0.99	A,B
	Halophila ovalis (Iriomote-type)	ITS	−3.44	0.95	0.99	A,B
	Cymodocea serrulata	ITS	−3.34	0.99	0.99	A,B
	Halodule uninervis	ITS	−3.39	0.97	1.00	A,B
Seagrass (temperate and subarctic zone)	*Zostera marina*	ITS	−3.40	0.97	0.99	A,B
	Zostera caulescens	ITS	−3.24	1.04	0.99	A,B
	Phyllospadix iwatensis	ITS	−3.31	1.01	1.00	A,B
Phaeophyceae (single species)	*Undaria pinnatifida*	ITS	−3.51	0.93	0.99	B
	Saccharina japonica	ITS	−3.42	0.96	1.00	B
	Saccharina longissima	COI	−3.34	0.99	0.99	A,B
	Ecklonia kurome	ITS	−3.42	0.96	1.00	A,B
	Sargassum horneri	ITS	−3.49	0.94	0.99	A,B
Phaeophyceae, Rhodophyta and Chlorophyta (Species complex)	*Sargassum* spp.	ITS	−3.47	0.94	0.99	A,B
	Bactrophycus spp.	ITS	−3.40	0.97	0.99	A,B
	Ulva spp.	ITS	−3.40	0.97	0.99	A,B
	Neopyropia spp.	ITS	−3.40	0.97	1.00	A,B
	Ecklonia/Eisenia spp.	ITS	−3.37	0.98	0.99	A,B
	Saccharina spp.	ITS	−3.21	1.05	0.99	A,B
Internal standard	*Crassostrea zhanjiangensis*	COI	−3.46	0.95	0.99	A,B
	Ammodytes hexapterus	CYTB	−3.48	0.97	0.99	A,B
DNA polymerase	*A:SsoFast Probes Supermix with ROX, B:iQ Multiplex Powermix*					

3.5. Application of the Designed qPCR System to Field Samples

The effect of PCR inhibitors or the matrix effect [42] was not detected in the DNA extracted from the sediment samples from Hiroshima Bay.

Environmental DNA from *Z. marina* and subgenus *Bactrophycus* spp. was detected in high concentrations in the sediments of almost all survey sites; in contrast, the subgenus *Sargassum* spp. was found off the northern shore of Yashirojima Island, and its eDNA was only detected close to this habitat (Figure 3).

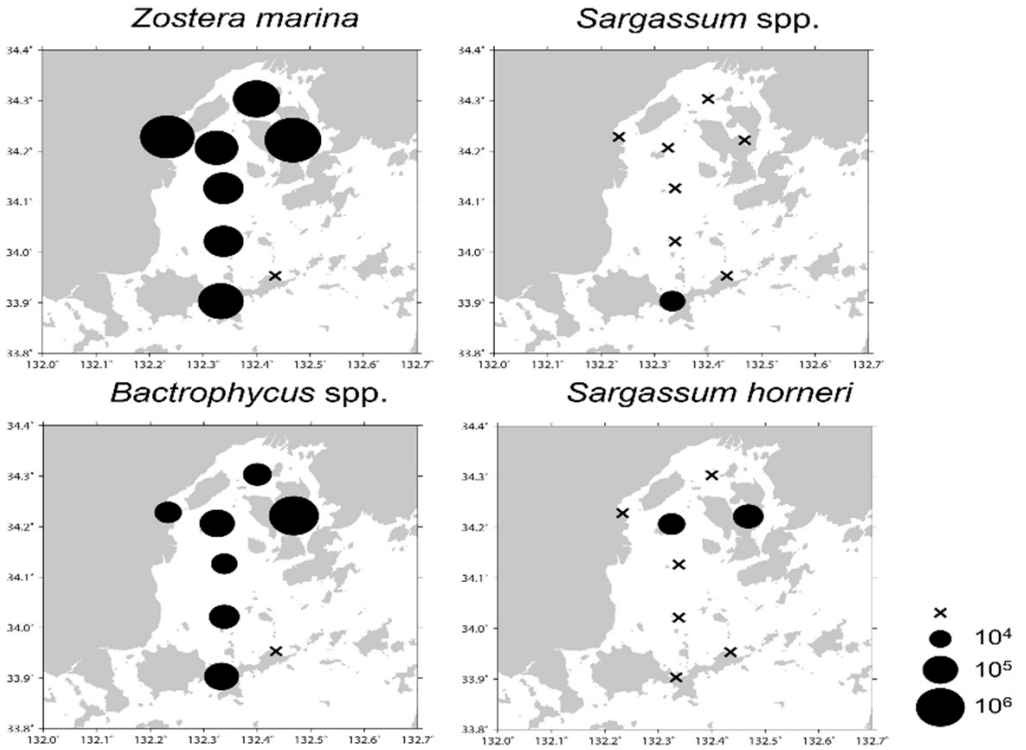
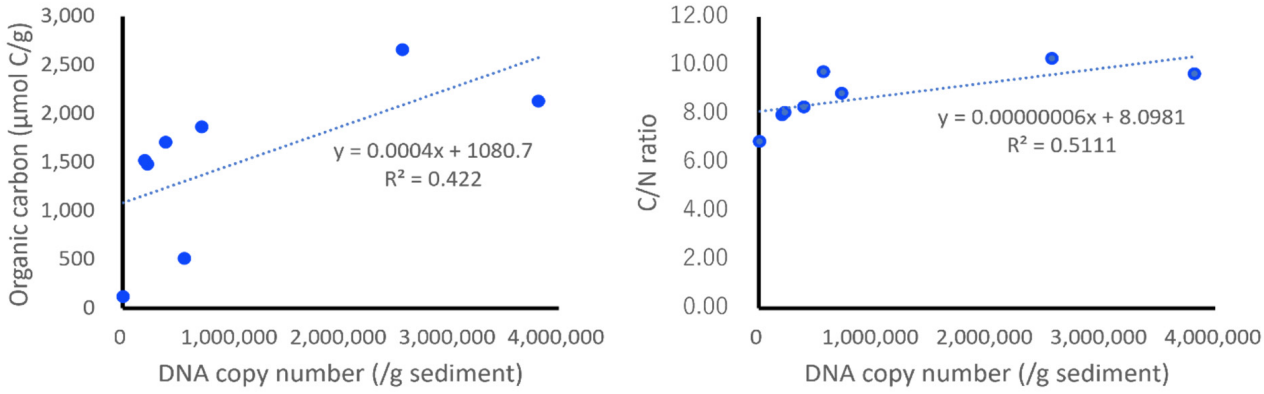


Figure 3. DNA copy number per g of marine sediment, according to sampling site: circles denote the relative amount, and 'x' symbols mark the sampling sites.

Measurements of the physical and chemical properties of the sediment from Hiroshima Bay are presented in Table 7. The correlation between these properties and the number of DNA copies detected in *Z. marina* and the subgenus *Bactrophycus* spp., which had the highest number of eDNA detections, was examined. The DNA copy number of the subgenus *Bactrophycus* spp. Did not correlate with the measured properties of the sediment, whereas the DNA copy number of *Z. marina* correlated with organic carbon content (Pearson two-sided test: r = 0.650, not significant) and C/N ratio (Pearson two-sided test: r = 0.715, $p < 0.05$) in the sediment samples (Figure 4). The data shown in Table 7 were used to calculate the origin of organic carbon in the bottom sediments, following the method of Miyajima et al. [44], using a 4-source model and a 3-source model. The results of the correlation analysis with the 3-source model, which is considered highly reliable, using the eDNA of eelgrass are shown in Figure 5. There was a low correlation between the amount of organic carbon derived from *Z. marina*, as calculated by the 3-source model (Table 8), and the detection results of eDNA (Pearson two-sided test: r = 0.463, not significant). From the quantification results of the eDNA of the four species or species groups shown in Figure 3, the detection and quantification limits were calculated using the calculation method described here. The values of LOD and LOQ, respectively, were: 2.2 DNA copies/μL and 14.8 DNA copies/μL for subgenus *Sargassum* spp.; 2.5 DNA copies/μL and 16.2 DNA copies/μL for subgenus *Bactrophycus* spp.; 2.4 DNA copies/μL and 19.4 DNA copies/μL for *Sargassum horneri*; and 2.0 DNA copies/μL and 10.5 DNA copies/μL for *Z. marina*. In this study, the amount of eDNA in the sediments of Hiroshima Bay was calculated based on the results of this calculation, and the data above the limit of quantification was used.

Table 7. Physical and chemical properties of the sediments in Hiroshima Bay.

Sampling Site	Sampling Position		Total Nitrogen (µmol N/g)	Organic Coarbon (µmol C/g)	C/N Ratio	Nitrogen Stable Isotope Ratio (d15N)	Carbon Stable Isotope Ratio (d13C)	Specific Surface Area (m^2/g)	Total Pore Volume (cm^3/g)	Average Pore Diameter (nm)
	Longitude	Latitude								
HSd-001	34-13.3	132-28.1	220	2,130	9.66	7.75	−21.29	26.02	0.119	18.30
HSd-003	34-18.2	132-24.0	210	1,863	8.87	8.07	−21.04	33.99	0.146	17.16
HSd-005	33-57.2	132-26.1	17	117	6.87	9.11	−19.95	3.60	0.015	16.45
HSd-007	33-54.2	132-20.0	52	512	9.76	7.21	−21.74	5.64	0.028	19.84
HSd-009	34-01.3	132-20.3	190	1,517	7.98	7.85	−20.86	32.09	0.123	15.39
HSd-011	34-07.6	132-20.3	183	1,479	8.07	7.84	−20.82	31.71	0.113	14.24
HSd-013	34-12.4	132-19.5	206	1,705	8.30	7.93	−20.85	35.03	0.110	12.58
HSd-015	34-13.7	132-14.0	258	2,658	10.30	6.19	−22.46	31.84	0.106	13.29



(**A**) (**B**)

Figure 4. Correlation analysis between the number of copies of the eDNA of *Zostera marina* and the amount of organic carbon (**A**) and the C/N ratio (**B**) in marine sediment samples from Hiroshima Bay.

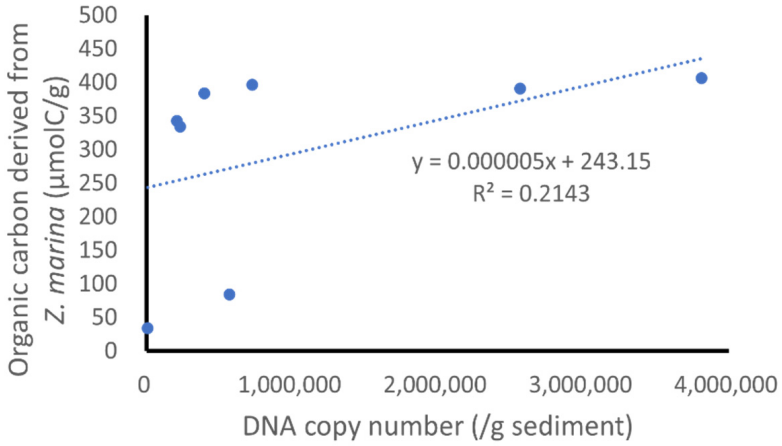


Figure 5. Correlation analysis between the amount of eelgrass *Zostera marina*-derived organic carbon (calculated using the three-parameter method of Miyajima et al. [44]) in marine sediment samples from Hiroshima Bay, and the eelgrass eDNA copy number.

Table 8. The amount of *Zostera marina*-derived organic carbon, calculated by the three-parameter method of Miyajima et al. [44] in the seabed sediments of Hiroshima Bay.

Sampling Site	Organic Carbon Derived from *Z. marina* (μmol C/g)			
	4-Source Model	SD*	3-Source Model	SD*
HSd-001	253	185	407	211
HSd-003	235	168	397	175
HSd-005	19	12	34	10
HSd-007	56	41	84	50
HSd-009	200	140	343	141
HSd-011	195	136	334	139
HSd-013	225	157	384	162
HSd-015	255	189	391	231

SD* means Standard deviation.

4. Discussion

4.1. Recommended Reaction System

4.1.1. Points to Note in the Design of Detection Systems for qPCR

The DLPs and PCR primers designed in this study (Tables 1–5) can be used in combination with duplex or multiplex if the reaction temperature is the same and if the fluorescent dyes that are labeled are different. The recommended reaction system is to correct the reactivity between wells by ROX, use duplex PCR for the detection of seaweeds, and use internal standards if necessary. We used the DLPs and PCR primers shown in Table 5 in combination with the fluorescent dyes, FAM and HEX, respectively, at the same reaction temperature; the following duplex combinations were used to analyze the marine sediment samples from Hiroshima Bay: subgenera *Sargassum* and *Bactrophycus* species; *Ulva* spp. and *Neopyropia* spp.; and *Ecklonia/Eisenia* spp. and *Saccharina* spp. These combinations allow for up to three DLPs and PCR primers. However, because these were designed within the short ITS region, non-specific reactions may occur depending on the combination, so careful consideration should be given to the choice of combination. When performing duplex or multiplex qPCR, it is desirable to consider the DLP and PCR primer to be used from the earliest design stage. In addition to conventional software, such as Primer 3 [47], there are also design services provided by pharmaceutical companies [48] that can be used for designing DLPs and PCR primers. Therefore, it is necessary to carefully consider which ones to combine.

4.1.2. Which DNA Polymerase and Real-Time PCR Instruments to Use?

Hamaguchi et al. [27] compared two types of DNA polymerases and explained the reaction results of each qPCR; even so, the quantitative nature of qPCR differs depending on the DNA polymerase used. Therefore, we believe that the same DNA polymerase should be used for setting the reaction conditions (e.g., reaction temperature), confirming the species specificity, preparing the calibration curve, and conducting quantitative analysis of field samples. In this study, we used two different types of SsoFast Probes Supermix with ROX and iQ Multiplex Powermix, manufactured by Bio-Rad, according to the method used by Hamaguchi et al. [27]. As depicted in Table 6, for some DLPs and PCR primers (*Rhizophora mucronate*, *Bruguiera gymnorrhiza*, *Saccharina japonica* and *Undaria pinnatifida*), only iQ Multiplex Powermix met the quantitative criteria established in this study.

In addition, depending on the real-time PCR system used, only two fluorescent dyes may be detected. However, because hydrolysis probes can detect multiple target genes simultaneously, it is desirable to use an instrument that can detect multiple fluorescent dyes at the same time to fully utilize their characteristics.

4.2. Detection of eDNA Derived from Mangroves

At least six species of mangroves are found in Japan: *Bruguiera gymnorhiza*, *Kandelia obovata*, *Rhizophora stylosa*, *Avicennia marina*, *Lumnitzera racemosa*, and *Sonneratia alba*, al-

though the mangrove palm *Nypa fruticans* is sometimes listed as a seventh species [49]. Lo et al. [50] reported that *Rhizophora apiculata* was also present. The species with the highest biomass on the islands of Ishigaki and Iriomote were *B. gymnorhiza* and *R. stylosa*; *A. marina*, *L. racemose*, and *S. alba* were found in small amounts in limited areas. Mangroves have a strong carbon dioxide sequestration effect and are an important taxon among blue carbons [51]. Miyajima et al. [52] used the qPCR system designed in this study to examine the sea sediments around the islands of Ishigaki and Iriomote, a location in Japan where mangroves thrive; mangrove-derived eDNA was detected in both offshore and deep-sea sediments. These results suggest that mangroves may act as a source of blue carbon by storing carbon dioxide in the system and transporting and accumulating its leaves, bark, and branches. Hence, mangroves not only store carbon in their vegetation but also accumulate it by transporting it to leaves and trees in other places and may function as a blue carbon. This finding was also suggested by Ortega et al. [19], and the present results confirm it.

4.3. Detection of eDNA Derived from Seagrasses

The qPCR system for subtropical seagrasses designed in this study has been used by Miyajima et al. [52] to analyze the environmental DNA of seafloor sediments off the islands of Ishigaki and Iriomote where these seagrasses grow. As a result, the eDNA of *Enhalus acoroides* and *Cymodocea rotundata* was detected even from sediments that were more than 1000 m deep, yet the eDNA of the sympatric *Thalassia hemprichii* was not detected. This may be because the degradation process differs among these three species, with *T. hemprichii* being the most easily degraded. However, green turtles feed on seagrasses (mainly *E. acoroides* and *C. rotundata* [53]), and the population of this species on the islands of Ishigaki and Iriomote has been increasing in recent years [54]; hence, the turtles are possibly implicated in the irregular dispersal of eDNA in this area.

Conversely, *Z. marina*, which is widely distributed from the temperate to the subarctic region of Japan, is closely related to *Z. caulescens* and is difficult to identify by the rbcL and matK regions of plastid DNA [55]. Therefore, the PCR primers and DLPs for the matK region were not designed for these species in this study. Furthermore, those used by Hamaguchi et al. [27] could not be employed to discriminate between *Z. marina* and *Z. caulescens*, as that study was conducted in the Seto Inland Sea, where *Z. caulescens* does not occur. In this study, the PCR primers and DLPs for *Z. marina* were redesigned based on the ITS region so that they would not react with *Z. caulescens*, which, likewise, grows in the study area.

4.4. Detection of eDNA Derived from Phaeophyceae, Rhodophyta and Chlorophyta

The qPCR system is usually designed for the accurate identification of a specific species. For mangroves and subtropical seagrasses and seagrasses and seaweeds in southwestern Japan, we constructed a species-specific qPCR system. However, numerous species of seaweeds grow in Japanese waters, and we expected that it would be difficult to follow the dynamics of their degradation products for each individual species by qPCR. Therefore, we designed DLPs and PCR primers to group species together that are taxonomically close and have similar ecological characteristics, and so examine them all together.

For example, the genus *Sargassum* has been divided into four clades, based on the sequence of the ITS region of nuclear DNA, as reported by Stiger et al. [34]. Among them, the species most widely distributed in the temperate zone in Japan are classified in the subgenus *Bactrophycus*, and the species distributed in the subtropical zone in the southern part of the temperate zone are classified in the subgenus *Sargassum*. In this study, we designed DLPs and PCR primers that could discriminate between these two subgenera, based on the difference in the ITS gene; accordingly, by using these, we were able to clearly identify eDNA in the sediments of Hiroshima Bay for the genus *Sargassum*. In the results of the analysis, we inferred how the algal bodies of the subgenus *Sargassum* (which have a limited distribution) are dispersed and deposited on the spatial scale of Hiroshima Bay after the plants are detached from the substrate.

Large kelp species that inhabit the seas off northern Japan have been used for food since ancient times and are traded at high prices as a foodstuff, supporting Japanese food culture. Because the price of kelp varies depending on where it is harvested, it is subdivided according to morphological information and is then given a scientific name. As a result of recent classifications based on genetic information, the number of cases that do not match the conventional classification is increasing, and the scientific names are frequently changed [56], causing taxonomic confusion. However, recent advances in chloroplast DNA and the total genome analysis of kelp species have led to genetically informed taxonomic studies [57,58]. Moreover, the temperate kelp species of *Ecklonia* and *Eisenia* are grown along the west coast of Japan, but their classification is changing as new species are being proposed, based on genetic information [59,60].

We used the available genetic information and designed DLPs and PCR primers for the species complex of *Saccharina*, *Ecklonia* and *Eisenia*, which are the most abundant genera of kelp in Japan.

The genera *Ulva* (green algae) and *Neopyropia* (red algae) are also confusingly classified, respectively [61–66]. Therefore, we constructed a qPCR system, with *Ulva pertusa* and *Neopyropia yezoensis* as the main species complex.

Moreover, in Japan, seaweed is widely cultivated for food; therefore, in designing the qPCR system, the farmed species were also considered. According to statistical information from the wild and cultured seaweed industry, the wild harvest of seaweeds in Japan in 2020 was 63,500 metric tons, of which kelp was the largest component, at more than 70% of the total harvest. In contrast, seaweed cultivation in 2020 produced 396,800 metric tons, of which the largest amount was *N. yezoensis*, followed by *Undaria pinnatifida* and *Saccharina* spp.; together, these three species accounted for 93.8% of the total amount farmed. Therefore, these wild-harvested and cultured species should be evaluated as another blue carbon resource; accordingly, we considered these species in the qPCR system. Kelp, in particular, has attracted attention for the blue-carbon function of the seaweed itself and for the blue-carbon function of kelp-derived dissolved organic carbon that is released during the growth process [67].

The DLPs and PCR primers for qPCR that are introduced here will be improved in the future, through implementation and verification of the results. In addition, it will be necessary to set a detection limit for the sake of quantitative improvements of the method.

4.5. Application of the Designed qPCR System to Field Samples

Analysis of the eDNA of seaweeds and seagrasses in the sediments of Hiroshima Bay revealed that *Zostera marina* and the subgenus *Bactrophycus* spp. were distributed throughout the bay (Figure 2) and were detected as eDNA in the sediments at most survey sites (Figure 3). *Ulva* spp. were widely distributed in Hiroshima Bay, especially in the tidal flats in the northern part of the bay, where they sometimes occur in large amounts and have large biomass [68]; even so, the eDNA of *Ulva* spp. could not be detected at all the study sites with >95% confidence, so the concentrations could not be calculated. One reason for this may be that *Ulva pertusa*, which is thought to be the largest component of *Ulva* spp., is easily degraded in seawater and does not remain as a persistent material in marine sediments. The application of the system is also meant to reveal which macrophytes have high blue carbon storage potential, and which ones do not. The results of this study indicate that when evaluating the blue carbon effect of the seaweeds found in Hiroshima Bay, we should focus on *Z. marina* and the subgenus *Bactrophycus* spp., and not necessarily on *Sargassum horneri* and the genus *Ulva* spp.

The seagrass *Z. marina* is known to be effective in accumulating and depositing allochthonous organic matter, in addition to its own vegetation. Unlike other seaweeds, seagrasses also contain cellulose and other components that are not easily degraded; therefore, their decomposition is slower and remains in the sediment for longer [13,45,69,70]. It is believed that there was a vast *Z. marina* bed in Hiroshima Bay until circa 1500 CE; however, the seagrass beds have clearly decreased because of land reclamation and coastal development.

The environmental DNA of *Z. marina*, detected in the sediments of Hiroshima Bay in this study, may have originated from past eelgrass beds and should be examined in detail.

In contrast, *S. horneri* is thought to comprise the majority of the subgenus *Bactrophycus* spp. in the Seto Inland Sea, where it is the seaweed species with the largest biomass [71]. Though it is an annual species, its annual production is predictably enormous, owing to high biomass. However, the detection rate of environmental DNA from this species in the sediments of Hiroshima Bay was low in this study. The reason for this may be that this species is highly buoyant, and, after withering and dying, it does not decompose in Hiroshima Bay but instead moves to other locations as drift algae. The species is also known as drifting seaweed, with deposits found on the deep-sea bottom near Japan [72]. On the other hand, *Sargassum macrocarpum*, *S. thunbergii*, and *S. muticum* belonged to the subgenus *Bactrophycus* spp. growing in Hiroshima Bay [73]. The present results suggest that these species may be decomposing in the Bay after the algal bodies have detached from the substrate, rather than being transported as drift algae to other areas, as is the case with *S. horneri*. This is also indicated by the results of the present study for the subgenus *Sargassum* spp. Therefore, although there is an impression that the genus *Sargassum* moves as drift algae, it is possible that more species are decomposed in situ after the algal bodies detach from the substrate. Therefore, the genus *Sargassum* may be related to the sequestration of organic carbon as blue carbons, in scales such as inland seas and bays in the extreme coastal regions, and the fate of individual species after the algal bodies have detached from the substrate should be investigated in detail in the future. The "Blue Carbon" report [1] states that the sedimentation of large brown algae has not been assessed, and, therefore, their blue carbon effect is unknown; our observations suggest that the contribution of brown algae, such as the subgenus *Bactrophycus* spp. and kelp *Saccharina* spp., to blue carbon should indeed be considered. Furthermore, the calculated values of blue carbon in the seas around Japan may be underestimated, as the assessment by Bertram et al. [4], for example, does not include these seaweeds. In the future, the contribution of large brown algae, such as the subgenus *Bactrophycus* spp. and kelps *Saccharina* spp., to the blue carbon assessment around Japan merits detailed investigation. In addition, the sediments of Hiroshima Bay were surveyed only once in our study, yet Wei et al. [74] reported seasonal variations in fish-derived eDNA in the sediments of Tokyo Bay, which is a similar estuary to Hiroshima Bay. If annual surveys were conducted in Hiroshima Bay, it is possible that the results would differ from the present results.

Environmental DNA is frequently used to search for rare and endangered species [12,14,16,75–77]. In Hiroshima Bay, *Sargassum carpophyllum*, *S. patens* and *S. piluliferum* are known to occur in limited areas. Many subtropical species are included in the genus *Sargassum*; if the water temperatures in the seas around Japan rise due to global warming, rare or newly invasive species from tropical areas might be found. For instance, Hamaguchi et al. [78,79] reported invasive subtropical oyster species in the seas around Japan. Therefore, it is expected that the locations where seaweeds thrive will likewise change due to rising water temperatures, and eDNA technology could also be effective in studying changes in the distribution of seaweeds.

Investigations of the dynamics of decomposing macrophytes using eDNA technology are considered useful, barring one major problem. To assess blue carbon storage or blue carbon stock, it is necessary to have a high correlation between organic carbon and eDNA. In a previous research example, the correlation between organic carbon and eDNA was high for the long-term, stable *Z. marina* beds in the Seto Inland Sea [27], but the correlation was low in almost all the other areas considered. The incompatibility between eDNA and organic carbon has been reported by Reef et al. [18] and Queirós et al. [7]; therefore, we considered this the biggest impediment to using eDNA for blue carbon assessment. To solve this problem, the introduction of methods other than eDNA, which is thought to have a high correlation with organic carbon content, is being considered (as summarized by Reich et al. [80] and Geraldi et al. [81]).

At present, we consider that the most appropriate way to identify blue carbon storage or blue carbon stock is to use a combination of comprehensive analysis using NGS and species-specific qPCR, considering the aforementioned advantages and disadvantages of these methods. Above all, qualitative and quantitative analyses of metabarcoding through NGS, using internal standards, have recently been reported [21], and we expect that more blue carbon storage or blue carbon stock will be successful, using metabarcoding through NGS for quantitative analysis. However, using this method to identify macrophyte species that contribute to the blue carbon release may be inappropriate because primers targeting the V7 and V9 regions of 18S rRNA also contain information derived from other organisms. Therefore, we are planning to design special primers, such as that for MiFish [82], to study macrophytes more efficiently.

4.6. Future Research Focus and Concepts

To detect the eDNA from marine macrophytes, one major question is what genetic regions to use. Hamaguchi et al. [27] designed DLPs and PCR primers in the ITS region of eelgrass nuclear DNA and the matK region of chloroplast DNA, to compare the two regions. First, they reported that the detection of these two regions differed, depending on the site of the eelgrass and also the extinction in marine sediments. Hamaguchi et al. [27] also used amplicons of 90 bp and 102 bp for the ITS and matK regions, respectively, which are different in length. Peixoto et al. [17] pointed out that different amplicon sizes may cause differences in the degradation process. Therefore, the amplicon size should be the same when making comparisons, whether this is because of the length of both PCR products or because the number of copies of each DNA in the cell or the location of each DNA must be clarified through studies using experimental degradation of eelgrass. In this study, we designed DLPs and PCR primers for the ITS region of nuclear DNA and the matK region or psbA region [83] of chloroplast DNA in mangroves and subtropical seagrasses, as well as in *Z. marina*, and used them to analyze eDNA in marine sediments around the islands of Ishigaki and Iriomote. However, when we analyzed the eDNA of seafloor sediments around the islands using these primers, the detection results of both primers did not coincide with each other but instead diverged significantly. This result warrants discussion in a future study; therefore, only the results of the ITS region of nuclear DNA were presented in this article.

Though it was otherwise not discussed here, to examine the correlation between eDNA from the marine macrophytes from which it was derived, we thought that a gene region with a small number of copies or a single copy would be easier to convert than a region with many copies, such as the ITS region. We thought that it would be easier to convert the ITS region into a single-copy gene region. Fortunately, single-copy genes have been reported in angiosperms [84–86], and we designed our DLPs and PCR primers using a part of the cellulose synthase genes [84] that are assumed to be single-copy genes in mangroves and seagrasses. We also designed DLPs and PCR primers using some regions of cellulose synthase that are assumed to be single-copy in mangroves and seagrasses. Using this method, we analyzed the angiosperms collected at Ishigaki and Iriomote, and marine sediment samples from around the two islands, and found low species specificity and low detectability in the marine sediments. These results suggest that single-copy gene regions may be unsuitable for the detection of eDNA. Thus, we believe that further investigation is needed to determine which gene region should best be used for the detection system of eDNA as a way to verify the fate of macrophytes after they wither and die in the marine environment. We expect that various problems will be encountered when using the DLPs and primers proposed here to analyze field samples, but that improvements will be achieved despite such setbacks.

Japanese people have long used seagrasses and seaweeds for food [87], fuel [88], and fertilizer [89], and some seaweeds, such as kelp, are essential ingredients in Japanese cuisine [56]. For this reason, seaweed cultivation is carried out in many parts of Japan [90], and it is necessary to discuss the evaluation of such seaweed cultivation when assessing

carbon dioxide sinks [91,92]. The cultivation of seaweeds has been increasing worldwide in recent years, for food, as a raw material for biochemicals, and for the production of bioethanol [92]. Nobutoki et al. [93] have suggested that the seaweeds utilized as energy, food, and biomass should be named "blue resources"; attempts are being made to add them to the list of blue carbon for their carbon sequestration effects. Moreover, the habitats of mangrove forests, salt marshes, seagrass beds, and tidal flats have been continuously lost because of coastal development, despite their important ecological functions in terms of biodiversity and material cycles [94]. Therefore, we have been conducting research to support the conservation of these ecosystems, and we believe that the concept of blue carbon is beneficial for promoting this [95].

5. Conclusions

This article introduces our qPCR technology for the analysis of eDNA to identify various marine macrophytes found in Japanese waters and presents the results of a field survey in Hiroshima Bay using the method.

The present results demonstrate that it was possible to identify the species of seaweeds that constitute blue carbon in coastal sediment. It was also found that the degradation process in the marine environment differs depending on the type of macrophyte. As described above, qPCR technology is useful for the evaluation of blue carbon in different ecosystems; future research should strive to combine the advantages of comprehensive analysis using NGS and this method of analysis, with high species specificity, using qPCR.

Author Contributions: M.H. (Masami Hamaguchi) carried out the molecular analysis on all the specimens and drafted the manuscript. T.M. carried out the pre-treatment of marine sediment samples collected from Hiroshima Bay, biochemical analysis of the marine sediment samples and drafted the manuscript. H.S. and M.H (Masakazu Hori) carried out the species identification of collected various macrophytes in this study, data analysis and drafted the manuscript. All authors collected specimens at various collection sites in Japan. All authors have read and agreed to the published version of the manuscript.

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