

1 **Technical note: Development of an extraction protocol and**

2 **colorimetric analysis for alginate in marine sediment**

3 Sota Nakazato¹, Takashi Kawahara¹, Reiji Ichii¹, Shingo Akita¹, Akira Inoue¹, Masaki Fujita¹, Hideyuki
4 Kurihara¹, Mitsuhiro Nakaya¹, and Atsushi Ooki¹

5 ¹Hokkaido University, Faculty of the Graduate School of Fisheries Sciences, 3-1-1 Minato Cho, Hakodate, Hokkaido 0418611,
6 Japan

7 *Correspondence to:* Sota Nakazato (nakazato.sota44@gmail.com)

8 **Abstract.** In this study, we developed a novel extraction method for alginate, a major organic component derived from brown
9 algae, in marine sediments and evaluated its applicability for quantitative analysis. Alginate analytical methods have been
10 established in food chemistry: we modified these techniques to apply them to marine sediments, which are characterized by
11 the cation composition (e.g., Ca, Mg, Fe) and humic substance-like high-molecular-weight organic compounds. By modifying
12 the protocol through the addition of EDTA-2Na and the omission of ethanol precipitation, we improved the extraction
13 efficiency of alginate from marine sediments, as demonstrated by spike recovery tests where recoveries of Na⁺-Alg
14 significantly increased from 38.7 % with the conventional method to 64.7–82.6 %. Enzymatic degradation tests using alginate
15 lyase confirmed that a portion of the extracted uronic acids from marine sediments (at least 34 %) was derived from alginate,
16 verifying its presence in the natural samples. Using this modified method, alginate contents in sediment samples from coastal
17 waters around Hokkaido, Japan, were quantified as 6.11–26.0 mg m⁻² in Funka Bay, 39.0–41.3 mg m⁻² in Hakodate Bay,
18 11.8–14.7 mg m⁻² off Cape Esan, and 58.3–74.1 mg m⁻² off Muroran. However, these values may be overestimated due to the
19 presence of other uronic acids; therefore, they should be interpreted with caution rather than being directly equated with
20 absolute alginate content.

21 **1 Introduction**

22 The role of marine plants in promoting carbon sequestration in the ocean interior, and hence their role in mitigating global
23 warming, has attracted increasing attention. The carbon sequestration potential of marine plants, particularly seaweeds,
24 mangroves, and salt marshes, is collectively referred to as “blue carbon” (Nellemann et al., 2009). Recently, the contribution
25 of macroalgae to blue carbon has gained attention as an effective approach to reducing atmospheric CO₂ levels (Hill et al.,
26 2015). Recent studies using environmental DNA, collection of macroalgal debris with trawls, and stable carbon isotope
27 analyses have provided evidence for the transport of organic matter derived from macroalgae to the deep ocean (Miyajima et
28 al., 2022; Wei et al., 2012; Fischer and Wiencke, 1992). Among the three major groups of macroalgae—brown, red, and

29 green—brown algae account for approximately half of the total macroalgal biomass and are the dominant group, particularly
30 in temperate and subpolar coastal oceans (Cai et al., 2021; Steneck et al., 2002). Brown algae contain a polysaccharide, alginate,
31 which is composed of uronic acids and accounts for 20–30 % of their dry weight (Usov et al., 2001). In this study, we focused
32 on alginate as a major organic component derived from brown algae.

33 Uronic acids are monosaccharides characterized by a terminal carboxyl group ($-\text{COOH}$); four primary types with distinct
34 stereochemical configurations—glucuronic, galacturonic, mannuronic, and guluronic acids—are commonly reported in the
35 marine environment (Bergamaschi et al., 1999), where they form various polysaccharides through glycosidic linkages.
36 Alginate is a linear polysaccharide composed of two epimeric uronic acid residues, β -D-mannuronic acid [M] and α -L-
37 guluronic acid [G], which form various block structures (MM, GG, and MG blocks) (Yang et al., 2011). Although also
38 produced by certain bacteria, alginate is primarily derived from the cell walls and intercellular matrix of brown algae
39 (Szekalska et al., 2016), which is considered to be predominant source in the marine environment from a biomass perspective.
40 Another major uronic-acid-containing polysaccharide is pectin, primarily composed of polymerized galacturonic acid
41 (Gerschenson, 2017). Pectin is produced by a wide range of terrestrial and marine plants, including several types of
42 phytoplankton that contribute significantly to marine primary production (Domozych et al., 2007). Both alginate and pectin
43 form gels through ionic interactions between their carboxy groups and polyvalent cations (Ca^{2+} , Cu^{2+} , and Fe^{3+}), whereas
44 monovalent cations such as Na^+ and K^+ promote solubilization. In particular, alginate forms significantly more rigid and
45 mechanically stable gels through the "egg-box" model compared to the weaker structures formed by pectin (Grant et al., 1973;
46 Fang et al., 2008).

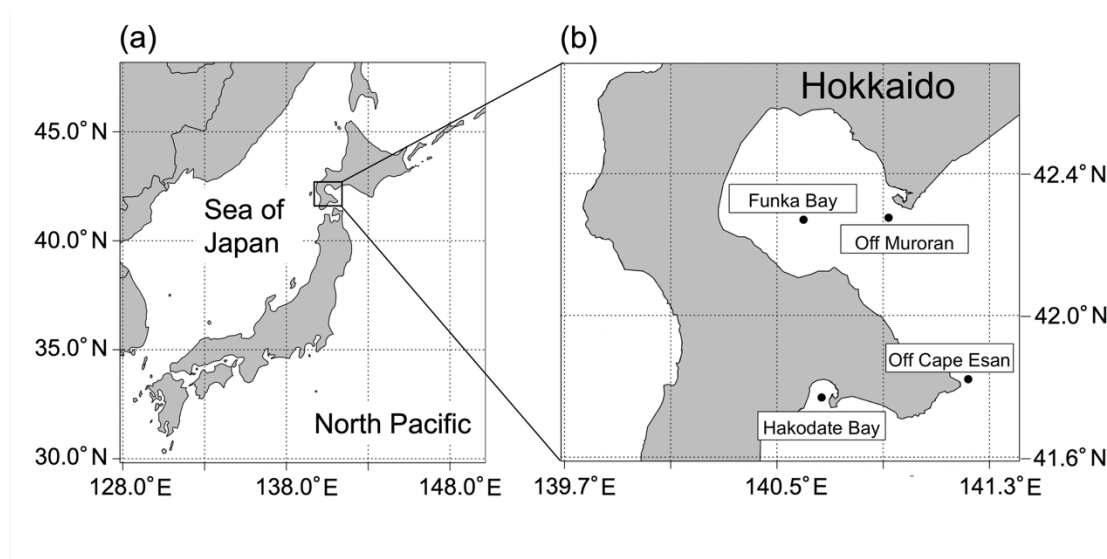
47 In brown algae cells, alginate primarily exists as various alginate salts (e.g., Ca^{2+} , Na^+ , Mg^{2+} , and K^+), with these cations
48 derived from seawater (Usov and Zelinsky, 2013). While brown algae are considered a significant source of organic carbon,
49 direct evidence of their contribution to carbon transport into the deep ocean remains limited. Although analytical methods for
50 alginate have been established for food samples (Kawasaki et al., 1998), their application to marine sediments can be hampered
51 by the complex matrix, including the cation composition, the trace levels of alginate present, and the coexistence of other
52 polyuronic acids like pectin. Particularly in the pore water of ocean sediments, high concentrations of iron ions interact with
53 alginate to form stable, insoluble iron (III) alginate (Fe^{3+} -Alg) gel, which can significantly inhibit extraction efficiency
54 (Klinkhammer, 1980; Menakbi et al., 2016). In this study, our aim was to develop a novel method for quantifying alginate in
55 marine sediment, based on conventional food analysis techniques, and to apply this method to samples collected from the
56 continental shelf to the shelf slope.

57 **2 Materials and methods**

58 **2.1 Sediment sample collection**

59 Ocean sediment was collected in December 2023 and March, August, October, and December 2024 from the seafloor of
60 Funka Bay, Hokkaido, Japan (Fig. 1). Funka Bay is a semi-enclosed bay with a maximum depth of 96 m, and brown algae

61 inhabit the shore of the bay. In the bay, subarctic water dominates from early spring (March) to middle summer (August), and
62 subtropical water enters in autumn (September–November) (Ohtani and Kido, 1980). Sediment samples were collected using
63 a grab sampler with a mouth area of 900 cm² (30 cm × 30 cm). The surface layer (0–1 cm depth) of the soft mud sediment was
64 collected in a plastic bag and stored in a freezer (−20 °C). Additional ocean sediment samples were collected in June 2024
65 from Hakodate Bay (30 m depth; soft mud sediment) and from the continental shelf slope in the coastal Pacific off Hokkaido
66 (off Cape Esan; 300 m depth; sandy mud sediment), and in February 2025 from off the coast of Muroran (off Muroran; 20 m
67 depth; soft mud sediment), using the same procedure, equipment, and storage conditions as those used for Funka Bay ones.
68



69
70 **Figure 1: Sampling sites in Funka Bay, Hakodate Bay, off Cape Esan, and off Muroran. (a) Geography of Japan's islands; (b)**
71 **sampling sites around Hokkaido.**

72 73 **2.2 Colorimetric analysis of alginate by the *m*-hydroxydiphenyl method**

74 Estimation of alginate content is commonly conducted using colorimetric methods that quantify uronic acids because of their
75 simplicity and ease of application. These methods typically rely on the formation of colored complexes between uronic acids
76 and specific reagents, such as carbazole and *m*-hydroxydiphenyl (Kumar and Kumar, 2017). We measured alginate in the
77 extracted sample with a colorimetric method, the *m*-hydroxydiphenyl method, following the procedure of Hung and Santschi
78 (2001). Hung and Santschi (2001) used sulfamate to reduce the interference of browning neutral carbohydrates; however, it
79 has been reported that addition of sulfamate can suppress the color development of uronic acids (Filisetti-Cozzi and Carpita,
80 1991). Given the potential reduction in color development when measuring trace amounts of alginate in marine sediments, in
81 this study, the sulfamate addition was omitted from the original protocol of Hung and Santschi (2001) to improve sensitivity.
82 Two reagents were used in the colorimetric analysis of alginate.

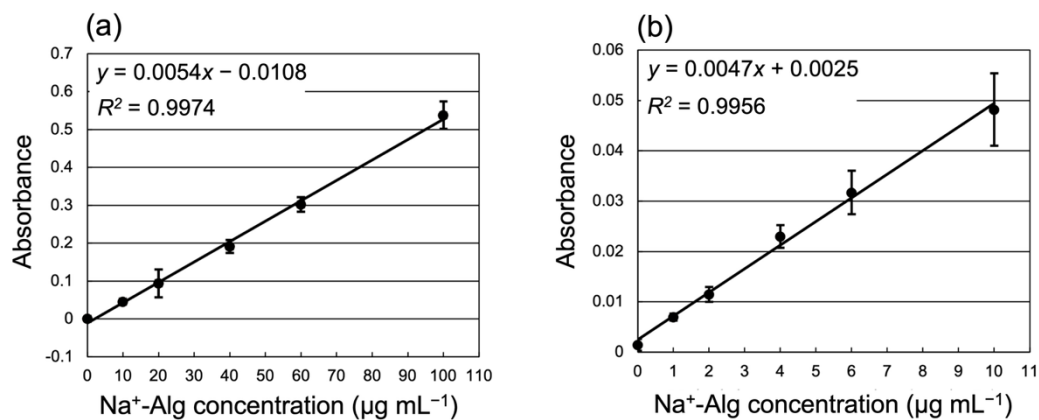
83 Reagent A: a 75 mmol L⁻¹ sodium tetraborate decahydrate (Wako, Osaka, Japan, purity ≥ 99 %) was prepared in concentrated
84 sulfuric acid (H₂SO₄, Wako, purity ≥ 95 %).

85 Reagent B: 1.5 g L⁻¹ 3-phenylphenol (Sigma-Aldrich, St. Louis, USA, purity 85 %) was prepared in 5.0 g L⁻¹ sodium hydroxide
86 (NaOH, Wako, purity ≥ 97 %) solutions. The reagent B container was covered with aluminum foil and stored in a refrigerator.

87 A 0.80 mL aliquot of sample solution was pipetted into a centrifuge tube, then 4.8 mL of reagent A was added to the tube.
88 The sample was manually mixed in an ice-water bath for 1 min to cool it to room temperature (25 °C). Subsequently, the tube
89 was heated at 100 °C for 10 min in a boiling water bath (7.0 L) and was then immediately cooled in the ice-water bath. After
90 cooling to room temperature, 60 μL of reagent B was added to the tube and mixed gently. After 5 min, the absorbance was
91 measured at 525 nm with a 1 cm cell using a spectrophotometer (U-2900, HITACHI, Tokyo, Japan). This colorimetric assay
92 was performed simultaneously on six samples within a single measurement run.

93 Standard solutions (0, 10, 20, 40, 60, and 100 μg mL⁻¹) were freshly prepared for each analysis using a sodium alginate
94 reagent (Na⁺-Alg, 500–600 cP, Wako, 1st grade) and Milli-Q water. A calibration curve for Na⁺-Alg (0–100 μg mL⁻¹) is shown
95 in Fig. 2a. The regression equation we obtained was 'y = 0.0054x - 0.0108'. Several blank samples (Milli-Q water added with
96 Reagents A and B) were also measured (n = 5). We determined the lower limit of quantification as a sample absorbance equal
97 to 10 times the standard deviation of the blank sample (stdev = 0.00040, n = 5), which corresponds to 0.0040 abs (= 10-fold
98 stdev) and 0.74 μg mL⁻¹. To confirm the quantitative accuracy near the lower limit, a calibration curve for low concentrations
99 of alginate (0, 1.0, 2.0, 4.0, 6.0, and 10 μg mL⁻¹) was prepared (Fig. 2b). The regression equation for low concentrations was
100 'y = 0.0047x + 0.0025'. The slopes of the calibration curve differed depending on the concentration range; thus, we used
101 separate regression equations for the low (0–10 μg mL⁻¹) and high (10–100 μg mL⁻¹) concentration ranges.

102



103 **Figure 2: Calibration curves of Na⁺-Alg measurement by the colorimetric method. (a) High concentrations (10–100 μg mL⁻¹, n = 5);**
104 **(b) low concentrations (0–10 μg mL⁻¹, n = 4). Error bars indicate one standard deviation.**
105

106

107 **2.3 Alginate extraction by a conventional food chemistry protocol**

108 We prepared three sediment samples, each with a wet weight of 300 g, from muddy sediment rich in organic material collected
109 from Funka Bay (Fig. 1) in December 2023. The weight of 300 g corresponds approximately to the amount of sediment material
110 obtained from the upper 0–1 cm layer using a grab sampler. The test samples ($n = 3$) were treated by the conventional alginate
111 extraction method used in food chemistry (Kawasaki et al., 1998; Udagawa et al., 2013), as follows (Steps 1–7).

112 **Preparation of test sediment samples:**

113 To prepare test samples, 500 mL of Milli-Q water and 20 mL of a 10 g L^{-1} sodium alginate ($\text{Na}^{\text{+}}\text{-Alg}$) solution containing 200
114 mg of $\text{Na}^{\text{+}}\text{-Alg}$ were added to 300 g of each sediment sample ($n = 3$) in a beaker, followed by stirring for 5 min. These samples
115 were labeled “sediment + $\text{Na}^{\text{+}}\text{-Alg}$ 200 mg”. The resulting suspensions were stored overnight (12 h) at $4 \text{ }^{\circ}\text{C}$ to allow the added
116 $\text{Na}^{\text{+}}\text{-Alg}$ to transform into naturally occurring alginate forms, such as magnesium alginate ($\text{Mg}^{2\text{+}}\text{-Alg}$), $\text{Ca}^{2\text{+}}\text{-Alg}$, and $\text{Fe}^{3\text{+}}\text{-Alg}$
117 gel. Although previous studies have not clearly identified the chemical forms of alginate in marine sediments, we hypothesized
118 that ion exchange would occur between $\text{Na}^{\text{+}}\text{-Alg}$ and $\text{Mg}^{2\text{+}}$, $\text{Ca}^{2\text{+}}$ or $\text{Fe}^{3\text{+}}$ ions, all of which are abundant in seawater and the
119 pore water of ocean sediments (Masuzawa and Kitano, 1983; Klinkhammer, 1980). The rationale for the selective interaction
120 of $\text{Fe}^{3\text{+}}$ —but not $\text{Fe}^{2\text{+}}$ —with alginate to form a gel is discussed in Sect. 3.2.1. After this treatment, alginate in the samples was
121 presumed to exist as a mixture of gelled forms ($\text{Ca}^{2\text{+}}\text{-Alg}$ and $\text{Fe}^{3\text{+}}\text{-Alg}$) and soluble salts ($\text{Na}^{\text{+}}\text{-Alg}$ and $\text{Mg}^{2\text{+}}\text{-Alg}$).

122 **Step 1 Enzymatic treatment to remove cellulose, lipids, proteins, and pectin:**

123 To remove impurities such as soluble sugars, proteins, and pectin from the sediment sample, enzymatic treatment was
124 employed. A total of 0.80 g of each of the following four enzymes was sequentially added to the sediment suspension: amylase
125 (KOKULASETM, Mitsubishi Chemical, Tokyo, Japan); cellulase (SCLASETMC, Mitsubishi Chemical); protease
126 (KOKULASETMP Granule, Mitsubishi Chemical); and pectinase (SCLASETMN, Mitsubishi Chemical). Each enzymatic
127 reaction was performed for 30 min under optimized conditions according to the manufacturer's specifications: amylase at $27 \text{ }^{\circ}\text{C}$
128 and pH 6.0; cellulase at $50 \text{ }^{\circ}\text{C}$ and pH 6.0; protease at $50 \text{ }^{\circ}\text{C}$ and pH 5.5; and pectinase at $38 \text{ }^{\circ}\text{C}$ and pH 4.2. A hot stirrer was
129 used to maintain the reaction temperature during stirring. The pH of each reaction suspension was adjusted as necessary by
130 adding 1.0 mol L^{-1} hydrochloric acid (HCl) and 3.0 mol L^{-1} NaOH solution. Following this enzymatic treatment, alginate in
131 the suspension sample was presumed to exist as a mixture of gelled forms ($\text{Ca}^{2\text{+}}\text{-Alg}$ and $\text{Fe}^{3\text{+}}\text{-Alg}$) and soluble salts ($\text{Na}^{\text{+}}\text{-Alg}$
132 and $\text{Mg}^{2\text{+}}\text{-Alg}$). Pectin in the sample was presumed to have been enzymatically degraded into monosaccharides, primarily
133 galacturonic acid.

134 **Step 2 Gelation of $\text{Na}^{\text{+}}\text{-Alg}$ and acidification to remove non-targeted compounds:**

135 To convert soluble alginate ($\text{Na}^{\text{+}}\text{-Alg}$ and $\text{Mg}^{2\text{+}}\text{-Alg}$) into $\text{Ca}^{2\text{+}}\text{-Alg}$ gel in the sediment suspension, 100 mL of 100 g L^{-1}
136 calcium chloride (CaCl_2 , Wako, purity $\geq 95 \%$) solution was added to the sample and stirred for 5 min. Subsequently,
137 approximately 15 mL of 5.0 mol L^{-1} HCl was added to adjust the pH to 1.0, and the suspension was kept overnight at room

138 temperature (25 °C). After the reaction, the resulting approximately 900 mL suspension was divided into eight 45 mL
139 centrifuge tubes and centrifuged (3500 rpm, 10 min, 2380 × g). The supernatant from each tube was carefully removed, and
140 40 mL of 0.10 mol L⁻¹ HCl was added to each tube. The tubes were manually shaken to ensure thorough mixing, followed by
141 centrifugation to remove the acidified supernatant. This acid treatment and the subsequent removal of the supernatant served
142 to eliminate polyvalent cations (e.g., Ca²⁺ and Mg²⁺), acid-soluble phenolic compounds, and easily degradable polysaccharides
143 such as fucoidans from the sediment sample (Bertagnolli et al., 2014). During this process, a portion of the Ca²⁺-Alg was
144 presumed to have undergone ion exchange with H⁺, forming insoluble H⁺-Alg (McHugh et al., 2001). Following this step,
145 alginate in the sediment sample was presumed to exist as a mixture of gelled forms (Ca²⁺-Alg and Fe³⁺-Alg) and protonated
146 alginate (H⁺-Alg) in a gelatinous form. Pectin was presumed to have been removed with the supernatant, because
147 depolymerized pectin is generally considered unable to form stable gel networks through Ca²⁺ crosslinking.

148 **Step 3 Alkaline extraction to dissolve alginate gel:**

149 Most of the sediment containing alginate gels in each tube was transferred to a single beaker using a spoon. To recover any
150 remaining precipitate in tubes, all eight tubes were rinsed with 500 mL of 10 g L⁻¹ sodium bicarbonate (NaHCO₃, Wako, purity
151 ≥ 99.5 %) solution, and the rinse solution was collected into a beaker. The resulting suspension in the beaker was heated to
152 60 °C with continuous stirring for 1.5 h, followed by reaction overnight at room temperature to convert the alginate gel to Na⁺-
153 Alg. Following this step, alginate in the suspension sample was presumed to exist primarily as soluble Na⁺-Alg; however, if
154 Fe³⁺-Alg was not soluble under the alkaline conditions (pH 8.0) of the NaHCO₃ solution, it may have remained in a gel form,
155 as described in Sect. 3.2.1.

156 **Step 4 Gelation of Na⁺-Alg into Ca²⁺-Alg:**

157 The pH of the suspension was adjusted to 5.0 by adding an appropriate volume of 1.0 mol L⁻¹ HCl. After centrifugation, the
158 supernatant was collected into a beaker. Subsequently, 100 mL of 300 g L⁻¹ CaCl₂ solution was added to the supernatant, and
159 the mixture was kept overnight at room temperature to convert soluble Na⁺-Alg to Ca²⁺-Alg gel. Following centrifugation, the
160 supernatant was removed, and the resulting precipitate was retained in the tubes. Then, an excess amount of Ca²⁺ was added
161 to completely react with the CO₃²⁻ ions derived from the previously added NaHCO₃ solution, thereby ensuring efficient ion
162 exchange between Na⁺-Alg and Ca²⁺. After this step, alginate in the precipitate sample was presumed to exist as Ca²⁺-Alg (gel
163 form).

164 **Step 5 Hydrochloric acid washing to remove oligosaccharides:**

165 Twenty milliliters of 0.10 mol L⁻¹ HCl was added to each centrifuge tube containing the precipitates. The tubes were manually
166 shaken to ensure thorough mixing, followed by centrifugation to remove the acidified supernatant. This acid-washing treatment
167 was repeated until oligosaccharides were no longer detected in the supernatant. To verify the presence of oligosaccharides, the
168 phenol-sulfuric acid colorimetric method was employed: 1.0 mL of the supernatant was transferred to a centrifuge tube, to
169 which 1.0 mL of 50 g L⁻¹ phenol (Wako, purity ≥ 99 %) solution was added and mixed. Subsequently, 5.0 mL of H₂SO₄ was

170 quickly added and mixed thoroughly. After 10 min, the appearance of a yellow color in the solution, indicating the presence
171 of oligosaccharides, was monitored. If color was observed, an additional round of acid-washing treatment was performed.
172 When the solution remained colorless, confirming the absence of detectable oligosaccharides, the acid-washing treatment was
173 terminated. The precipitates from each centrifuge tube were then collected into a single beaker using a spoon. After this HCl
174 treatment, alginate in the precipitate sample was presumed to exist as a mixture of Ca^{2+} -Alg (gel form) and H^+ -Alg (gelatinous
175 form).

176 **Step 6 Collection of Na^+ -Alg:**

177 One hundred milliliters of 10 g L^{-1} NaHCO_3 solution was added to a beaker containing the precipitates. The suspension was
178 then heated to $60 \text{ }^\circ\text{C}$ with continuous stirring for 1.5 h, followed by reaction overnight at room temperature to convert Ca^{2+} -
179 Alg to Na^+ -Alg. After centrifugation, the supernatant was collected into a clean beaker. Following this step, alginate in the
180 solution sample was expected to exist as soluble Na^+ -Alg. This extract solution appeared dark brown, likely due to the presence
181 of residual phenolic compounds (McHugh, 1987).

182 **Step 7 Ethanol precipitation to remove residual non-targeted compounds:**

183 To remove non-targeted compounds soluble in organic solvents, such as pigmentation, fats, and phenolic compounds, 400 mL
184 of ethanol (Wako, purity: 99.5 %) was added to the beaker containing the extract solution (Saji et al., 2022; Trica et al., 2019).
185 The ethanol–water mixture was kept overnight at room temperature to precipitate alginate, because alginate is insoluble in
186 ethanol (Gomez et al., 2009). The resulting precipitate was collected by filtration using a polycarbonate membrane filter
187 (Nuclepore, Whatman, Maidstone, UK; pore size: $10 \text{ }\mu\text{m}$, diameter: 47 mm). The filter with the retained precipitate was
188 transferred to a beaker and air-dried overnight at room temperature. Subsequently, 100 mL of 10 g L^{-1} NaHCO_3 solution was
189 added to the beaker containing the dried filter, and the mixture was stirred for 1.0 h. The solution was then kept overnight at
190 room temperature to ensure complete dissolution of Na^+ -Alg. After centrifugation, the supernatant was recovered; however, it
191 exhibited a slight brown coloration. To reduce the coloration and ensure compatibility with colorimetric analysis, the
192 supernatant was diluted 20-fold with Milli-Q water and analyzed colorimetrically as described in Sect. 2.2. However, if trace
193 amounts of Na^+ -Alg are soluble in ethanol, significant loss may occur during the extraction of trace levels of Na^+ -Alg from
194 ocean sediment, as described in Sect. 3.2.2.

195 **2.4 Collection of phytoplankton aggregates**

196 In the marine environment, phytoplankton are the dominant primary producers, and thus a substantial proportion of the organic
197 matter deposited on the seafloor is presumed to originate from them. Phytoplankton are also known to produce uronic acids,
198 including glucuronic acid derived from structural polysaccharides and galacturonic acid derived from pectin, both of which
199 are constituents of the cell wall (Bergamaschi et al., 1999; Gügi et al., 2015; Domozych et al., 2007). To ensure the accuracy

200 of the modified alginate extraction protocol described in Sect. 3.2.3, it was necessary to confirm that polyuronic acids other
201 than alginate are not detected from phytoplankton aggregate samples by the modified extraction protocol.

202 Phytoplankton aggregates were collected from the water column in Funka Bay (Hokkaido, Japan, Fig. 1) during the peak of
203 the spring phytoplankton bloom in March of 2018, 2019, and 2021. Sampling was conducted using a plankton net with a mouth
204 area of 707 cm² (mesh size: 300 μm; mouth diameter: 30 cm) towed from near the seafloor (87 m depth) to the surface. During
205 the bloom, the chlorophyll-a concentrations in the water reached approximately 20 μg L⁻¹. It has been reported that
206 approximately half of the annual primary production in Funka Bay occurs during the spring bloom, with a substantial portion
207 of diatom aggregates sinking and being deposited on the seafloor (Kudo et al., 2000). We assumed that the amount of
208 phytoplankton aggregates collected by the plankton net corresponded to the sedimented amount of polyuronic acids derived
209 from phytoplankton on the seafloor surface within the same area (30 cm diameter). The collected phytoplankton samples (*n* =
210 3) were processed using the modified extraction protocol (described in Sect. 3.2.3) and analyzed colorimetrically (as described
211 in Sect. 2.2) to evaluate the detectability of polyuronic acids derived from the phytoplankton samples.

212 **2.5 Verification of alginate assisted with alginate-degrading enzyme**

213 Colorimetric methods estimate alginate content by quantifying uronic acids; however, these methods may also detect uronic
214 acids derived from sources other than alginate, potentially leading to overestimation. To verify that the extracted material from
215 the ocean sediment was alginate, we employed an alginate lyase to confirm its presence through enzymatic degradation. The
216 alginate lyase used in this study was HULK (NIPPON GENE, Tokyo, Japan), which is the most effective enzyme for formation
217 of oligosaccharide from only alginate polymers.

218 HULK most preferably degrades MM blocks, by splitting glycosyl linkages of the alginate chain via the β-elimination
219 mechanism (Inoue et al., 2014). When alginate is present in the extract solution, the enzymatic reaction catalyzed by HULK
220 proceeds to degrade the alginate polymer into di- and trisaccharides. The addition of Ca²⁺ to di- and trisaccharides derived
221 from alginate does not induce gelation, because these low-molecular-weight fragments lack the chain length required for
222 effective crosslinking with Ca²⁺. Therefore, if the extract solution was treated with HULK and subsequently subjected to Ca²⁺
223 addition, a significant reduction in calcium-induced precipitation would indicate that the original extract had contained an
224 amount of alginate.

225 The analytical sample was 75 mL of the solution extracted from 1.8 kg of sediment collected off Muroran (Fig. 1), prepared
226 by using the modified extraction protocol (described in Sect. 3.2.3).

227 The extract solution appeared dark brown, similar in color to algal extracts rich in polyphenols as reported by
228 Andriamanantoanina and Rinaudo (2010), suggesting the possible presence of polyphenols in the sample. It has been reported
229 that polyphenols can form complexes with carbohydrates such as alginates and proteins, thereby inhibiting enzymatic activity,
230 and that the inhibition of alginate degradation by alginate lyase is correlated with the concentration of soluble polyphenols
231 (Moen et al., 1997). Therefore, it is essential to remove polyphenols from the sample prior to enzymatic degradation.

232 2.5.1 Bleaching treatment

233 Bleaching treatment has been reported to effectively remove pigments from alginate extracts derived from dark brown algae,
234 which are presumed to be rich in polyphenols, although direct quantification was not performed in that study
235 (Andriamanantoanina and Rinaudo, 2010). Therefore, in the present study, bleaching treatment was applied to reduce the
236 polyphenol content in the extract solution.

237 Three hundred and seventy-five milliliters of 24 mL L⁻¹ sodium hypochlorite (NaClO, Wako) solution was added to 75 mL
238 of extract solution in a beaker and stirred for 20 min. Subsequently, 188 mL of Milli-Q water was added, followed by
239 neutralization with 0.05 mol L⁻¹ HCl. Then, 20 mL of 300 g L⁻¹ CaCl₂ was added, and the mixture was kept overnight at room
240 temperature to convert soluble Na⁺-Alg to Ca²⁺-Alg gel. The following day, the precipitate was collected by centrifugation and
241 transferred to a clean beaker. Forty milliliters of 10 g L⁻¹ NaHCO₃ solution was added to the precipitate, and the suspension
242 was heated to 60 °C with continuous stirring for 1.5 h, followed by reaction overnight at room temperature to convert Ca²⁺-
243 Alg to Na⁺-Alg. Finally, the supernatant was collected by centrifugation. To reduce the coloration of the extract solution as
244 much as possible, this bleaching process was repeated six times with appropriate adjustments to the reagent quantities (Table
245 1). After the final bleaching step, the volume of the sample was reduced to 15 mL. The same procedure was also applied to 75
246 mL of 10 µg mL⁻¹ Na⁺-Alg solution.

247 2.5.2 Enzymatic degradation of alginate

248 To prepare the buffer solution for adjusting pH and salinity, 1.0 mL of 1.0 mol L⁻¹ Tris-HCl (pH 7.5, NIPPON GENE), 1.0
249 mL of 1.0 mol L⁻¹ Tris-HCl (pH 8.0, NIPPON GENE), 3.0 mL of 5.0 mol L⁻¹ sodium chloride (NaCl, Wako, purity ≥ 99.5 %)
250 solution, and 5.0 mL of Milli-Q water were combined in a tube and thoroughly mixed. For the enzymatic reaction, 5.0 mL of
251 Milli-Q water, 0.80 mL of the prepared buffer solution, 2.0 mL of the bleached extract solution, and 0.40 mL of alginate lyase
252 (HULK) were sequentially added to a centrifuge tube with gentle mixing after each addition. This enzymatic degradation
253 experiment was performed in triplicate using the same sample ($n = 3$). In parallel, a control experiment was performed without
254 the addition of alginate lyase. In this case, 5.0 mL of Milli-Q water, 0.80 mL of the prepared buffer solution, 2.0 mL of the
255 bleached extract solution, and 0.40 mL of Milli-Q water instead of alginate lyase were sequentially added to a centrifuge tube
256 with gentle mixing after each addition. The control experiment was also conducted in triplicate ($n = 3$).

257 The same enzymatic experiment was performed on the bleached Na⁺-Alg standard solution ($n = 1$). In addition, a blank sample
258 ($n = 1$) was prepared as a control using water in place of the extract solution: specifically, 5.0 mL of Milli-Q water, 0.80 mL
259 of the buffer solution, 2.0 mL of Milli-Q water, and 0.40 mL of alginate lyase (HULK) were sequentially added to a centrifuge
260 tube with gentle mixing. The mixtures were centrifuged (3500 rpm, 1 min, 2380 × g) to remove residual water from the inner
261 wall surface and subsequently incubated in an incubator (SLI-220, EYELA, Tokyo, Japan) at 30 °C for one week. After
262 incubation, the pH of each mixture was adjusted to 4.0 using 1.0 mol L⁻¹ HCl. Subsequently, 10 mL of 300 g L⁻¹ CaCl₂ was
263 added, and the mixtures were kept overnight at room temperature to convert Na⁺-Alg remaining in the mixture to Ca²⁺-Alg.

264 On the following day, the resulting precipitates were collected by filtration using polycarbonate membrane filters (Nuclepore,
 265 Whatman; pore size 0.40 μm , diameter 47 mm). At this stage, the oligomerized alginate produced by alginate lyase was
 266 expected to remain in the filtrate, because it does not form crosslinks in the presence of Ca^{2+} . Each filter containing the
 267 precipitate was transferred into a separate tube, to which 5.0 mL of 20 g L^{-1} NaHCO_3 solution was added. The tubes were then
 268 incubated in a 60 $^\circ\text{C}$ water bath for 1.5 h with gentle shaking every 10 min, followed by reaction overnight at room temperature
 269 to convert Ca^{2+} -Alg to Na^+ -Alg. The supernatants were collected by centrifugation (3500 rpm, 10 min, $2380 \times g$), and analyzed
 270 colorimetrically as described in Sect. 2.2.

271

272 **Table 1 Reagent quantities used in each repetition of the bleaching treatment**

Bleaching process run number	Sample (mL)	NaClO aq (mL)	Milli-Q water (mL)	CaCl_2 aq (mL)	NaHCO_3 aq (mL)
1	75.0	375	188	20.0	40.0
2	40.0	200	100	10.0	30.0
3	30.0	150	75.0	10.0	30.0
4	30.0	150	75.0	10.0	50.0
5	50.0	250	125	10.0	50.0
6	50.0	250 ^a	125	10.0	15.0

273 a: NaClO concentration was 36 mL L^{-1} for this experimental run only; the concentration in all other runs was 24 mL L^{-1} .

274

275 3 Results and discussion

276 3.1 Spike recovery of alginate using the conventional food chemistry method

277 Table 2 shows the yield of alginate extracted from the sediment sample and the corresponding spike recovery based on the
 278 conventional extraction method as used in food chemistry. The spike recovery of Na^+ -Alg from the sample was found to be
 279 36.8–40.7 %. These results suggest that the conventional extraction approach is not suitable for efficient recovery of alginate
 280 from ocean sediment.

281

282 **Table 2 Spike recovery of added Na^+ -Alg from ocean sediment by using the food extraction method^a**

Sample	Absorbance	Yield ($\text{mg per } 300 \text{ g}^{-1}$) ^b	Spike recovery ^c (%)
Sediment	0.209	81.41	40.7
+ Na^+ -Alg (200 mg)	0.197	76.96	38.5
	0.188	73.63	36.8
			average 38.7

283 a: Conventional extraction method used in food chemistry

284 b: 300 g indicates the sediment sample weight

285 c: Spike recovery = Yield (mg 200 mg⁻¹)

286

287 3.2 Modification of extraction treatment

288 3.2.1 Dissolve Fe³⁺-Alg present in the sediment sample

289 If Fe³⁺-Alg, formed via ion exchange between alginate and Fe³⁺, is present in a sediment sample, the gel may not dissolve in
290 the NaHCO₃ solution and could remain in the sediment even after alkaline treatment (Step 3), leading to reduced recovery. To
291 address this issue, we investigated the optimal conditions for the simultaneous dissolution of both Fe³⁺-Alg and Ca²⁺-Alg gels,
292 which are presumed to be the predominant forms of alginate present prior to the alkaline treatment. Disodium dihydrogen
293 ethylenediamine tetraacetic acid dihydrate (EDTA-2Na) is a potent chelator that forms more stable complexes with Fe³⁺ than
294 the binding affinity of Fe³⁺ to alginate (Berner and Hood, 1983). Because the stability of EDTA-metal complexes depends on
295 pH (Ueno, 1959), we investigated the dissolution behavior of Fe³⁺-Alg and Ca²⁺-Alg in EDTA-2Na solutions adjusted to pH
296 4.0 and 5.0, and compared the results with those obtained using NaHCO₃ solution at pH 8.0.

297 Fe³⁺-Alg gel was prepared by dropping 20 mL of 10 g L⁻¹ Na⁺-Alg solution into 20 mL of 100 g L⁻¹ iron (III) chloride
298 hexahydrate (FeCl₃, Wako, purity ≥ 99 %) solution in a beaker. To ensure complete reaction between Na⁺-Alg and Fe³⁺ ions,
299 the resulting Fe³⁺-Alg/Na⁺-Alg suspension was repeatedly filtered through a 100 μm stainless steel mesh using a silicone
300 spatula. The reddish Fe³⁺-Alg gel retained on the mesh was collected and rinsed three times with Milli-Q water. For comparison,
301 Ca²⁺-Alg gel was similarly prepared by dropping 20 mL of 10 g L⁻¹ Na⁺-Alg solution into 20 mL of 100 g L⁻¹ CaCl₂ solution.
302 Each gel type (Fe³⁺-Alg and Ca²⁺-Alg) was divided into two portions. One half of each was added to 500 mL of 10 g L⁻¹
303 NaHCO₃ solution and heated to 60 °C with continuous stirring for 1.5 h. The other halves were added to 500 mL of 80 g L⁻¹
304 EDTA-2Na (Kanto Chemical, Tokyo, Japan, purity > 99.5 %) solution, which had an initial pH of 4.0, and stirred for 1.5 h.
305 These suspensions were then kept overnight at room temperature. The presence or absence of remaining gel was assessed
306 visually. Subsequently, the pH of the EDTA-2Na solution was adjusted to 5.0 by adding 3.0 mol L⁻¹ NaOH solution. After
307 stirring for an additional 1.5 h, the mixtures were again kept overnight at room temperature, and the presence of gel was
308 assessed visually.

309 Most of the Fe³⁺-Alg did not dissolve in NaHCO₃ (pH 8.0) solution, but it did dissolve completely in EDTA-2Na solution at
310 pH 4.0 and pH 5.0 (Table 3). Ca²⁺-Alg, in contrast, was soluble in both NaHCO₃ (pH 8.0) and EDTA-2Na (pH 5.0) solutions,
311 but it remained insoluble in EDTA-2Na at pH 4.0. Based on these observations, EDTA-2Na solution at pH 5.0 was selected
312 as the optimal condition for the simultaneous dissolution of Fe³⁺-Alg and Ca²⁺-Alg in ocean sediment samples.

313

314 **Table 3 Results of dissolution tests on Fe³⁺-Alg and Ca²⁺-Alg in NaHCO₃ and EDTA-2Na solutions**

Sample	NaHCO ₃ (pH 8.0)	EDTA-2Na (pH 5.0)	EDTA-2Na (pH 4.0)
Fe ³⁺ -Alg gel	remained	dissolved	dissolved
Ca ²⁺ -Alg gel	dissolved	dissolved	remained

315

3.2.2 Omit ethanol precipitation in the final purification step

In the field of food chemistry, Na⁺-Alg is generally considered to be insoluble in ethanol–water mixtures; therefore, precipitation of Na⁺-Alg in a 4:1 (v/v) ethanol–water solution is commonly employed as the final step in extraction and purification. However, we were concerned that trace amounts of Na⁺-Alg might dissolve in a 4:1 (v/v) ethanol–water solution. If such minor solubility exists, it could result in significant loss during the extraction of trace levels of Na⁺-Alg from ocean sediment.

To evaluate the dissolution behavior, we prepared two Na⁺-Alg solutions (0.50 g L⁻¹ and 2.0 g L⁻¹; 100 mL each) and added 400 mL of ethanol (99.5 %) to each. The mixtures were kept overnight at room temperature to allow precipitation. Afterward, each mixture was filtered through a polycarbonate membrane filter (Nuclepore, Whatman; pore size: 0.22 μm, diameter: 47 mm). The filtrates were collected and evaporated to complete dryness. The resulting residues were re-dissolved in 20 mL of Milli-Q water. Subsequently, 0.50 mL of each re-dissolved sample was transferred to a separate tube and diluted 40-fold with Milli-Q water. The concentration of Na⁺-Alg in each diluted sample was then determined using the *m*-hydroxydiphenyl colorimetric method.

We observed that the 2.0 g L⁻¹ Na⁺-Alg solution was partially soluble in ethanol, with approximately 66 % dissolution, whereas the 0.50 g L⁻¹ Na⁺-Alg solution was completely dissolved (Table 4). These results indicate that trace amounts of alginate are soluble in ethanol. Consequently, a significant loss of alginate could occur during ethanol precipitation, particularly when extracting trace levels of alginate from ocean sediment. Therefore, the ethanol precipitation process was considered unsuitable for the extraction of alginate from sediment samples.

Table 4 Dissolved amounts and loss rates of Na⁺-Alg in an ethanol/water (4:1) solution

Na ⁺ -Alg sample (100 mL)	Na ⁺ -Alg in sample (g)	Dissolved amount (g)	loss rate
0.50 g L ⁻¹	0.050	0.057 ^a	100 %
2.0 g L ⁻¹	0.200	0.132	66 %

a: The dissolved amount (0.057 g) was determined to be higher than the original amount (0.050 g). This value is within the analytical precision (±15 %).

3.2.3 Modified extraction protocol

We modified two key aspects of the alginate extraction protocol. First, an EDTA-2Na treatment step was introduced to dissolve Fe³⁺-Alg and Ca²⁺-Alg gels into the liquid phase. Second, the ethanol precipitation step was omitted from the final purification process to avoid significant loss of alginate. The modified extraction protocol, in which Steps 1, 2, 4, and 5 remain identical to those described in the Methods section, is presented below. A complete overview of the updated protocol for alginate extraction from ocean sediment is provided in supplementary material (Fig. S1).

346 We prepared nine sediment samples with a wet weight of 300 g using muddy sediment rich in organic material collected from
347 Funka Bay (Fig. 1) in March 2024. The test samples ($n = 3 \times 3$) were treated by the modified alginate extraction method as
348 follows (Steps 1–7).

349 **Preparation of test sediment samples:**

350 To prepare the test samples, 500 mL of Milli-Q water and 20 mL of Na^+ -Alg solutions at concentrations of 10 g L^{-1} , 5.0 g L^{-1} ,
351 and 2.5 g L^{-1} —containing 200 mg, 100 mg, and 50 mg of Na^+ -Alg, respectively—were added to 300 g of sediment sample (n
352 = 3) in separate beakers, followed by stirring for 5 min. These samples were labeled as “sediment + Na^+ -Alg 200 mg,”
353 “sediment + Na^+ -Alg 100 mg,” and “sediment + Na^+ -Alg 50 mg,” respectively. The resulting suspensions were stored
354 overnight at 4°C to allow the added Na^+ -Alg to transform into Mg^{2+} -Alg, Ca^{2+} -Alg gel, and Fe^{3+} -Alg gel. After this treatment,
355 alginate in the samples was presumed to exist as a mixture of gelled forms (Ca^{2+} -Alg and Fe^{3+} -Alg) and soluble salts (Na^+ -Alg
356 and Mg^{2+} -Alg).

357 **Step 3 Alkaline extraction to dissolve alginate gel:**

358 To prepare 80 g L^{-1} EDTA-2Na solution, 40 g of EDTA-2Na was dissolved in 500 mL of Milli-Q water. The pH of the
359 resulting solution was 4.0 without any adjustment. Most of the sediment containing alginate gel from each tube was transferred
360 into a single beaker using a spoon. To recover any remaining precipitate in tubes, all eight tubes were rinsed with 500 mL of
361 80 g L^{-1} EDTA-2Na solution (pH 4.0), and the rinse solution was collected into a beaker. The resulting suspension in the
362 beaker was stirred for 1.5 h, followed by reaction overnight at room temperature to convert Fe^{3+} -Alg to Na^+ -Alg via chelation.
363 Subsequently, the pH of the suspension was adjusted to 5.0 by adding 3.0 mol L^{-1} NaOH solution, followed by stirring for
364 another 1.5 h and reaction overnight at room temperature to convert Ca^{2+} -Alg into Na^+ -Alg through ion exchange. Following
365 this step, alginate in the suspension sample was presumed to exist primarily as soluble Na^+ -Alg.

366 **Step 6 Collection of Na^+ -Alg:**

367 An appropriate volume (20–100 mL) of 10 g L^{-1} NaHCO_3 solution, adjusted based on the estimated amount of alginate, was
368 added to a beaker containing the precipitates to keep the alginate concentration within the range of the calibration curve (Fig.
369 2). For instance, 100 mL of 10 g L^{-1} NaHCO_3 solution was added to the sample that had been supplemented with alginate
370 during the preparation step, because it was presumed to contain a large amount of Ca^{2+} -Alg. Conversely, for the sample without
371 added alginate, which was presumed to contain a trace amount of Ca^{2+} -Alg, only 20 mL of 10 g L^{-1} NaHCO_3 solution was
372 used. The suspension was then heated to 60°C with continuous stirring for 1.5 h, followed by reaction overnight at room
373 temperature to convert Ca^{2+} -Alg to Na^+ -Alg. After centrifugation, the supernatant was collected into a clean bottle. Following
374 this step, alginate in the solution sample was expected to exist as soluble Na^+ -Alg.

375 **Step 7 Prepare the solution for colorimetric analysis:**

376 The extract solution appeared dark brown, likely due to the presence of residual phenolic compounds (McHugh, 1987). To
 377 reduce the coloration and ensure compatibility with colorimetric analysis, the extract solution was diluted 20–100-fold with
 378 Milli-Q water and analyzed colorimetrically as described in Sect. 2.2.

379 3.2.4 Spike recovery of alginate using the modified protocol

380 Table 5 shows the yield of alginate extracted from the sediment sample and the corresponding spike recovery based on the
 381 modified extraction protocol. We achieved higher spike recoveries (64.7–82.6 %) by using the modified extraction protocol
 382 than by the conventional protocol (recovery 38.7 %; Table 2). It should be noted that these efficiencies reflect the recovery of
 383 alginate naturally present in the sediment samples; therefore, the reported values may be slightly overestimated.

384
 385 **Table 5 Spike recovery of added Na⁺-Alg from the ocean sediment when using the modified extraction protocol^a**

Sample	Absorbance	Yield (mg 300 g ⁻¹) ^b	Spike recovery ^c (%)
Sediment	0.405	154.00	77.0
+ Na ⁺ -Alg (200 mg)	0.399	151.78	75.9
	0.435	165.11	82.6
			average 78.5
Sediment	0.179	70.30	70.3
+ Na ⁺ -Alg (100 mg)	0.164	64.74	64.7
	0.193	75.48	75.5
			average 70.2
Sediment	0.080	33.63	67.3
+ Na ⁺ -Alg (50 mg)	0.090	37.33	74.7
	0.092	38.07	76.1
			average 72.7

386 a: The modified protocol includes Fe³⁺-Alg dissolution and does not involve ethanol precipitation.

387 b: 300 g indicates the sediment sample weight.

388 c: Spike recovery = Yield (mg 200 mg⁻¹, 100 mg⁻¹, or 50 mg⁻¹)

389

390 3.3 Interference by polyuronic acid derived from phytoplankton

391 Table 6 shows the yield of uronic acid extracted from the phytoplankton aggregates by applying the modified extraction
 392 protocol. The average yield was 0.33 (mg sample⁻¹), corresponding to 23 % of the average uronic acid yield obtained from
 393 sediment samples collected in Funka Bay in August, October, and December 2024 (Fig. 1), as described in Sect. 3.5. These
 394 results indicate that although a fraction of the uronic acids (galacturonic acid or glucuronic acid) derived from phytoplankton
 395 remained in the extract solution, the modified extraction protocol effectively eliminated most of the interference from
 396 phytoplankton-derived polyuronic acids, thereby enabling the selective and efficient recovery of alginate.

397

398 **Table 6 Yield of uronic acid from plankton net samples**

Plankton net sample	Chlorophyll-a (g sample ⁻¹)	Modified extraction	
		Absorbance	Yield (mg sample ⁻¹) ^a
Funka Bay (2018.3)	4.93	0.006 ^b	0.30
Funka Bay (2019.3)	3.87	0.006 ^b	0.30
Funka Bay (2021.3)	3.57	0.007 ^b	0.38

399 Sample volumes for Funka Bay (2018 and 2019) were 800 mL, and for Funka Bay (2021) was 600 mL.

400 a: Yield (mg) of uronic acid was converted to Na⁺-Alg concentration using the calibration curve in Fig. 2b.

401 b: These absorbances exceed the lower limit (absorbance = 0.0040) described in Sect. 2.2.

402

403 3.4 Reduction in amount of alginate after enzymatic degradation

404 In Sect. 3.3, we demonstrated that a fraction of the uronic acids derived from phytoplankton remained after applying the
 405 modified extraction protocol. To confirm that the uronic acids present in the extract solution originated from alginate, we
 406 evaluated changes in uronic acid content following enzymatic degradation with alginate lyase.

407 The Na⁺-Alg concentration in the extract solution decreased from 18.11–18.67 μg mL⁻¹ (without enzyme) to 11.81–12.37 μg
 408 mL⁻¹ after enzymatic treatment (Table 7). A similar reduction was observed for the Na⁺-Alg standard solution, which decreased
 409 from 2.23 μg mL⁻¹ to 0.32 μg mL⁻¹ upon alginate lyase treatment. These results demonstrate that alginate present in both the
 410 extract and the standard solutions was effectively degraded by the enzyme, thereby supporting the presence of alginate in the
 411 extracted material.

412 However, the extract solution retained relatively high Na⁺-Alg concentrations (11.81–12.37 μg mL⁻¹) even after enzymatic
 413 treatment. This result suggests that the bleaching treatment was insufficient to completely remove polyphenols, which may
 414 have interfered with alginate lyase activity. In addition, the absorbance of the water sample treated with alginate lyase was
 415 0.004, indicating that residual enzyme in the measurement sample contributed slightly to the colorimetric reading; however,
 416 this effect was considered negligible. Taken together, these findings indicate that the organic matter extracted from ocean
 417 sediments using the modified protocol contained alginate, accounting for at least 34 % of the detected uronic acids.

418

419 **Table 7 Na⁺-Alg concentrations of extract solution, Na⁺-Alg standard solution, and Milli-Q water (blank), both with and without**
 420 **alginate lyase treatment**

Sample	Absorbance	Na ⁺ -Alg (μg mL ⁻¹)
Alginate lyase-treated extract solution	0.053	11.81
	0.054	12.00
	0.056	12.37
Untreated extract solution	0.090	18.67
	0.087	18.11
	0.088	18.30

Alginate lyase-treated Na ⁺ -Alg solution	0.004	0.32
Untreated Na ⁺ -Alg solution	0.013	2.23
Alginate lyase-treated water (blank)	0.004	-

421

422

423 3.5 Amount of alginate in the surface of ocean sediment in a coastal subarctic area

424 The modified extraction protocol was applied to the ocean sediment samples (300 g) collected from the surface 0–1 cm depth
 425 in the coastal area around southern Hokkaido, Japan (Fig. 1). Abundant alginate was detected in all extracted samples, with
 426 values of 0.55–6.67 mg 300 g⁻¹ (alginate/wet weight of sediment). In Funka Bay, alginate concentrations were 0.55–2.34 mg
 427 300 g⁻¹, corresponding to 6.11–26.0 mg m⁻² (alginate/surface sediment area) during the sampling period from August to
 428 December 2024. In Hakodate Bay and off Cape Esan, concentrations were 3.51–3.72 mg 300 g⁻¹ (39.0–41.3 mg m⁻²), and
 429 1.06–1.32 mg 300 g⁻¹ (11.8–14.7 mg m⁻²), respectively, during June 2024. Off Muroran, concentrations were 5.25–6.67 mg
 430 300 g⁻¹ (corresponding to 58.3–74.1 mg m⁻²) during February 2025. However, given that the extract solution may contain a
 431 fraction of phytoplankton-derived uronic acids and that confirmed alginate accounted for at least 34 % of the total, these
 432 detected values should be interpreted with caution rather than being directly equated with absolute alginate content.

433

434 **Table 8 Yield of alginate from ocean sediment samples**

Sample	Absorbance	Yield (mg 300 g ⁻¹) ^a
	0.015	1.06
Off Cape Esan (2024.6)	0.018	1.32
	0.016	1.15
Hakodate Bay (2024.6) ^b	0.019	3.51
	0.020	3.72
	0.014	0.98
Funka Bay (2024.8)	0.030	2.34
	0.014	0.98
	0.018	1.32
Funka Bay (2024.10)	0.010	0.64
	0.025	1.91
	0.009	0.55
Funka Bay (2024.12)	0.022	1.66
	0.030	2.34
	0.026	6.67
Off Muroran (2025.2)	0.023	5.82
	0.023	5.82
	0.021	5.25

435 a: The dilution rates of samples were 20-fold for those from off Cape Esan and Funka Bay, 50-fold for those from Hakodate Bay, and 100-
436 fold for those from off Muroran.
437 b: Due to the amount of sediment collected, the number of samples was two ($n = 2$).

438

439 **4 Conclusions**

440 In this study, we developed a novel extraction protocol for alginate in marine sediments, based on a conventional method used
441 in food chemistry, and evaluated its applicability for quantitative analysis. We modified two key aspects—the addition of
442 EDTA-2Na and the omission of ethanol precipitation—to improve the extraction efficiency of alginate from marine sediments.
443 Spike recovery tests with Na⁺-Alg (200, 100, and 50 mg) demonstrated substantially higher recoveries (64.7–82.6 %) by using
444 the modified protocol than with the conventional method (38.7 %). To assess potential interference from non-alginate
445 polyuronic acids, such as those derived from phytoplankton, phytoplankton aggregates were subjected to the modified protocol.
446 Although most of the interference was effectively eliminated, the minor amounts of uronic acids were detected, corresponding
447 to 23 % of the estimated alginate content in marine sediments at the same site. To confirm that the uronic acids in marine
448 sediments extracts originated from alginate, an enzymatic degradation test using alginate lyase was performed on the natural
449 extracts. The uronic acid concentration decreased following enzymatic degradation, confirming that a portion of the uronic
450 acids (at least 34 %) was derived from alginate. Finally, the alginate content of marine sediments collected around southern
451 Hokkaido, Japan, was quantified using the modified protocol. Alginate concentrations were 6.11–26.0 mg m⁻² in Funka Bay
452 (August–December 2024), 39.0–41.3 mg m⁻² in Hakodate Bay (June 2024), 11.8–14.7 mg m⁻² off Cape Esan (June 2024), and
453 58.3–74.1 mg m⁻² off Muroran (February 2025). These findings demonstrate the establishment of a quantification method for
454 alginate in marine sediments and provide an initial application to the surface sediments of the target area.

455 **Data availability**

456 The dataset supporting this study is available on Zenodo at <https://doi.org/10.5281/zenodo.19484055> (Nakazato, 2026).

457 **Author contribution**

458 SN designed the extraction protocol and conducted the experiments.

459 AO designed the research and conducted the observations.

460 TK and RI assisted with the experiments.

461 SA, AI, MF, HK, and MN provided advice on the research.

462 SN and AO prepared the manuscript with contributions from all co-authors.

463 **Competing interests**

464 The contact author has declared that none of the authors has any competing interests.

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