Technical note: Development of an extraction protocol and colorimetric analysis for alginate in marine sediment

Sota Nakazato[1], Takashi Kawahara[1], Reiji Ichii[1], Shingo Akita[1], Akira Inoue[1], Masaki Fujita[1], Hideyuki Kurihara[1], Mitsuhiro Nakaya[1], and Atsushi Ooki[1]

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

Correspondence to: Sota Nakazato (nakazato.sota.c4@elms.hokudai.ac.jp)

Abstract. Transportation of organic carbon from coastal macroalgae, particularly brown algae, to deeper ocean layers has recently attracted attention as an effective mechanism for carbon sequestration. However, no observational data are currently available on the amounts of organic carbon derived from brown algae in marine environments such as seawater or sediments. In this study, we developed an extraction protocol for alginate, a polysaccharide unique to brown algae that accounts for 20–30 % of their dry weight, to provide quantitative and direct evidence of brown algal carbon sequestered in ocean sediments. Alginate extraction and colorimetric analysis methods are well established in food chemistry: we modified these techniques to apply them to marine sediments, which are characterized by high concentrations of cations (e.g., Ca, Mg, Fe) and humic substance-like high-molecular-weight organic compounds. We applied this new method to sediment samples collected from coastal waters around Hokkaido, Japan. Alginate contents were quantified as 6.11–26.0 mg m^{-2} in Funka Bay, 39.0–41.3 mg m^{-2} in Hakodate Bay, 11.8–14.7 mg m^{-2} off Cape Esan, and 58.3–74.1 mg m^{-2} off Muroran.

1 Introduction

The role of marine plants in promoting carbon sequestration in the ocean interior, and hence their role in mitigating global warming, has attracted increasing attention. The carbon sequestration potential of marine plants, particularly seaweeds, mangroves, and salt marshes, is collectively referred to as "blue carbon" (Nellemann et al., 2009). Recently, the contribution of macroalgae to blue carbon has gained attention as an effective approach to reducing atmospheric CO_2 levels (Hill et al., 2015). The transport of organic matter derived from macroalgae from nearshore areas to the deep ocean has been investigated using various approaches, including collection of macroalgal debris with trawls (Wei et al., 2012), camera observations from remotely operated vehicles (Harrold et al., 2003), stable carbon isotope analyses (Fischer and Wiencke, 1992), and lipid analyses (Harvey and Kennicutt, 1992). Environmental DNA studies have provided direct evidence for the long-range transport of macroalgal material to the open ocean (Miyajima et al., 2022). Furthermore, by combining a global coastal ocean hydrographic model with a macroalgal habitat model, the annual carbon sequestration by macroalgae has been estimated at 56

29 Tg C yr^{-1} (Filbee-Dexter et al., 2024). Among the three major groups of macroalgae—brown, red, and green—brown algae

30 account for approximately half of the total macroalgal biomass and are the dominant group, particularly in temperate and

31 subpolar coastal oceans (Cai et al., 2021; Steneck et al., 2002). Brown algae contain a unique polysaccharide, alginate, which

32 is composed of uronic acids and accounts for 20–30 % of their dry weight (Usov et al., 2001). In this study, we focused on

33 alginate as the most dominant organic carbon derived from brown algae.

34 Uronic acids are monosaccharides in which the terminal primary hydroxy group (–CH$_2$OH), typically located at the C-6

35 position, is oxidized to a carboxylic acid (–COOH). In the marine environment, four types of uronic acids—glucuronic acid,

36 galacturonic acid, mannuronic acid, and guluronic acid—have been reported (Bergamaschi et al., 1999). These uronic acids

37 differ in the carbon backbone structure and the stereochemical configuration of their hydroxy groups. Uronic acids form

38 polysaccharides through glycosidic linkages, typically between the anomeric carbon (C-1) of one residue and the hydroxy

39 group at the C-4 position of another. Alginate is a linear polysaccharide consisting of two epimeric uronic acid residues (β-D-

40 mannuronic acid [M] and α-L-guluronic acid [G]), which form blocks of repeated M residues (MM blocks), repeated G residues

41 (GG blocks), and alternating M and G residues (MG blocks) (Yang et al., 2011). Alginate is primarily derived from the cell

42 walls and intercellular matrix of brown algae, including various macroalgae species, and is also produced by certain bacterial

43 strains (Szekalska et al., 2016). From a biomass perspective, brown algae are considered to be the predominant source of

44 alginate in the marine environment. Pectin is primarily composed of polymerized galacturonic acid, in which a portion of the

45 carboxy groups of galacturonic acid—a type of uronic acid—are methyl-esterified (Gerschenson, 2017). Pectin is known to

46 be produced by a wide range of plants, including both terrestrial and marine species. Notably, several types of phytoplankton,

47 which contribute markedly to marine primary production, have also been reported to produce pectin (Domozych et al., 2007).

48 Both alginate and pectin form gels through ionic interactions between their carboxy groups (–COOH) at the C-6 position and

49 polyvalent cations such as Ca^{2+}, Cu^{2+}, and Fe^{3+}, with the exception of Mg^{2+}. In contrast, the presence of monovalent cations

50 such as Na$^+$ and K$^+$ does not promote gelation and instead contributes to solubilization. Alginate forms mechanically strong

51 gels through crosslinking with Ca^{2+} ions, a process commonly described by the "egg-box" model (Grant et al., 1973). Although

52 pectin also forms gels upon interaction with Ca^{2+}, the resulting gels are generally weaker than calcium alginate (Ca^{2+}-Alg) gels,

53 primarily due to structural differences between the two polysaccharides (Fang et al., 2008).

54 In the cells of brown algae, alginate primarily exists as the Ca^{2+}, Na$^+$, Mg^{2+}, and K$^+$ salts of alginic acid, with these cations

55 derived from seawater (Usov and Zelinsky, 2013). In the marine environment, alginate gels—primarily Ca^{2+}-Alg—may sink

56 to deeper layers of the ocean. Although brown algae are considered to have substantial potential as a blue carbon source,

57 particularly in subpolar and polar coastal regions, direct evidence of organic carbon derived from brown algae being

58 transported to the deep ocean is lacking. Notably, alginate—produced exclusively by brown algae—has not been detected in

59 either seawater or marine sediments. To accurately assess the contribution of brown algae to blue carbon sequestration, it is

60 essential to quantify sedimented alginate on the ocean floor. Although methods for analyzing alginate from organic matter,

61 such as food samples, have been extensively developed (Kawasaki et al., 1998), a method for detecting alginate in marine

62 sediments has not yet been established. Marine sediments may differ from food samples in several respects, including their

63   cation composition, the trace levels of alginate present, and the coexistence of other polyuronic acids (such as pectin) in

64   addition to alginate. In this study, our aim was to develop a novel analytical method for quantifying alginate in marine sediment,

65   based on conventional food analysis techniques, and to apply this method to samples collected from the continental shelf to

66   the shelf slope.

67   **2 Materials and methods**

68   **2.1 Sediment sample collection**

69   Ocean sediment was collected in December 2023 and March, August, October, and December 2024 from the seafloor of

70   Funka Bay, Hokkaido, Japan (Fig. 1). Funka Bay is a semi-enclosed bay with a maximum depth of 96 m, and brown algae

71   inhabit the shore of the bay. In the bay, subarctic water dominates from early spring (March) to middle summer (August), and

72   subtropical water enters in autumn (September–November) (Ohtani and Kido, 1980). Sediment samples were collected using

73   a grab sampler with a mouth area of 900 cm^2 (30 cm × 30 cm). The surface layer (0–1 cm depth) of the soft mud sediment was

74   collected in a plastic bag and stored in a freezer (−20 °C). Additional ocean sediment samples were collected in June 2024

75   from Hakodate Bay (30 m depth; soft mud sediment) and from the continental shelf slope in the coastal Pacific off Hokkaido

76   (off Cape Esan; 300 m depth; sandy mud sediment), and in February 2025 from off the coast of Muroran (off Muroran; 20 m

77   depth; soft mud sediment), using the same procedure, equipment, and storage conditions as those used for Funka Bay ones.

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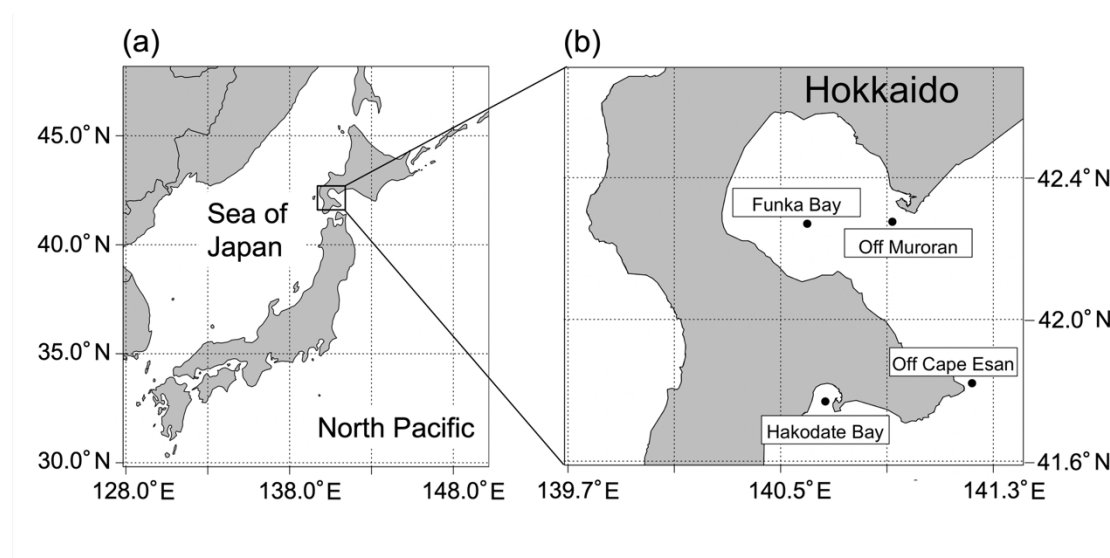
80   **Figure 1: Sampling sites in Funka Bay, Hakodate Bay, off Cape Esan, and off Muroran. (a) Geography of Japan's islands; (b)**
81   **sampling sites around Hokkaido.**

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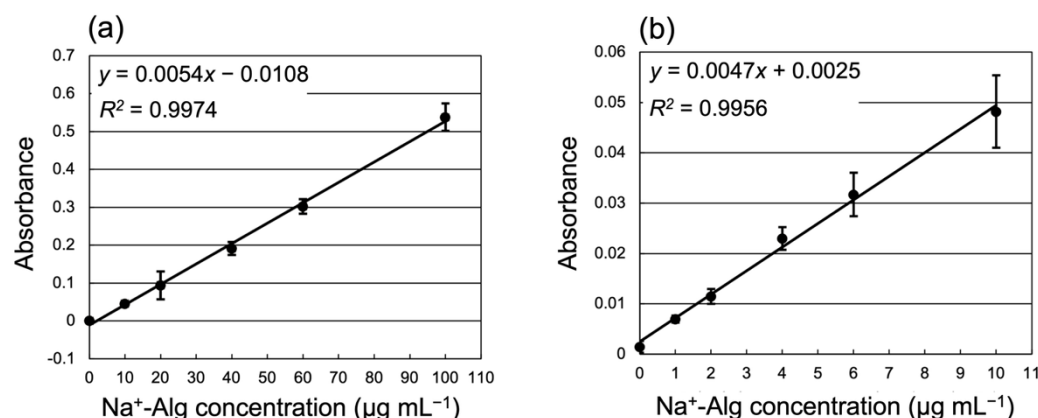
2.2 Colorimetric analysis of alginate by the *m*-hydroxydiphenyl method

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

Reagent A: a 75 mmol L^{-1} sodium tetraborate decahydrate (Wako, Osaka, Japan, purity $\geq$ 99 %) was prepared in concentrated sulfuric acid (H_2SO_4, Wako, purity $\geq$ 95 %).

Reagent B: 0.15% (w/v) 3-phenylphenol (Sigma-Aldrich, St. Louis, USA, purity 85 %) was prepared in 0.5 % (w/v) sodium hydroxide (NaOH, Wako, purity $\geq$ 97 %) solutions. The reagent B container was covered with aluminum foil and stored in a refrigerator.

A 0.8 mL aliquot of sample solution was pipetted into a centrifuge tube, then 4.8 mL of reagent A was added to the tube. The sample was manually mixed in an ice-water bath for 1 min to cool it to room temperature (25 °C). Subsequently, the tube 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 cooling to room temperature, 60 µL of reagent B was added to the tube and mixed gently. After 5 min, the absorbance was measured at 525 nm with a 1 cm cell using a spectrophotometer (U-2900, HITACHI, Tokyo, Japan). This colorimetric assay was performed simultaneously on six samples within a single measurement run.

Standard solutions (0, 10, 20, 40, 60, and 100 µg mL^{-1}) were freshly prepared for each analysis using a sodium alginate 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 in Fig. 2a. The regression equation we obtained was '$y = 0.0054x - 0.0108$'. Several blank samples (Milli-Q water added with Reagents A and B) were also measured ($n = 5$). We determined the lower limit of quantification as a sample absorbance equal to 10 times the standard deviation of the blank sample (stdev = 0.00037, $n = 5$), which corresponds to 0.0037 abs (= 10-fold stdev) and 0.069 µg mL^{-1}. To confirm the quantitative accuracy near the lower limit, a calibration curve for low concentrations 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 '$y = 0.0047x + 0.0025$'. The slopes of the calibration curve differed depending on the concentration range; thus, we used separate regression equations for the low (0–10 µg mL^{-1}) and high (10–100 µg mL^{-1}) concentration ranges.

114
115 **Figure 2: Calibration curves of Na$^+$-Alg measurement by the colorimetric method. (a) High concentrations (10–100 μg mL^{-1}, $n = 5$);**
116 **(b) low concentrations (0–10 μg mL^{-1}, $n = 4$). Error bars indicate one standard deviation.**

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118 **2.3 Alginate extraction by a conventional food chemistry protocol**

119   We prepared three sediment samples, each with a wet weight of 300 g, from muddy sediment rich in organic material collected
120   from Funka Bay (Fig. 1) in December 2023. The test samples ($n = 3$) were treated by the conventional alginate extraction
121   method used in food chemistry (Kawasaki et al., 1998; Udagawa et al., 2013), as follows (Steps 1–7).

122 **Preparation of test sediment samples:**

123   To prepare test samples, 500 mL of Milli-Q water and 20 mL of a 1.0 % (w/v) sodium alginate (Na$^+$-Alg) solution containing
124   200 mg of Na$^+$-Alg were added to 300 g of each sediment sample ($n = 3$) in a beaker, followed by stirring for 5 min. These
125   samples were labeled "sediment + Na$^+$-Alg 200 mg". The resulting suspensions were stored overnight (12 h) at 4 °C to allow
126   the added Na$^+$-Alg to transform into naturally occurring alginate forms, such as magnesium alginate (Mg^{2+}-Alg), Ca^{2+}-Alg gel,
127   and iron (III) alginate (Fe^{3+}-Alg) gel. Although previous studies have not clearly identified the chemical forms of alginate in
128   marine sediments, we hypothesized that ion exchange would occur between Na$^+$-Alg and Mg^{2+}, Ca^{2+} or Fe^{3+} ions, all of which
129   are abundant in seawater and the pore water of ocean sediments (Masuzawa and Kitano, 1983; Klinkhammer, 1980). The
130   rationale for the selective interaction of Fe^{3+}—but not Fe^{2+}—with alginate to form a gel is discussed in Sect. 3.2.1. After this
131   treatment, alginate in the samples was presumed to exist as a mixture of gelled forms (Ca^{2+}-Alg and Fe^{3+}-Alg) and soluble
132   salts (Na$^+$-Alg and Mg^{2+}-Alg).

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

134   To remove impurities such as soluble sugars, proteins, and pectin from the sediment sample, enzymatic treatment was
135   employed. A total of 0.80 g of each of the following four enzymes was sequentially added to the sediment suspension: amylase
136   (KOKULASE^{TM}, Mitsubishi Chemical, Tokyo, Japan); cellulase (SCLASE^{TM}C, Mitsubishi Chemical); protease

137 (KOKULASE™P Granule, Mitsubishi Chemical); and pectinase (SCLASE™N, Mitsubishi Chemical). Each enzymatic

138 reaction was performed for 30 min under optimized conditions according to the manufacturer's specifications: amylase at 27 °C

139 and pH 6.0; cellulase at 50 °C and pH 6.0; protease at 50 °C and pH 5.5; and pectinase at 38 °C and pH 4.2. A hot stirrer was

140 used to maintain the reaction temperature during stirring. The pH of each reaction suspension was adjusted as necessary by

141 adding 1.0 mol L^{-1} hydrochloric acid (HCl) and 3.0 mol L^{-1} NaOH solution. Following this enzymatic treatment, alginate in

142 the suspension sample was presumed to exist as a mixture of gelled forms (Ca^{2+}-Alg and Fe^{3+}-Alg) and soluble salts (Na^+-Alg

143 and Mg^{2+}-Alg). Pectin in the sample was presumed to have been enzymatically degraded into monosaccharides, primarily

144 galacturonic acid.

145 **Step 2 Gelation of Na^+-Alg and acidification to remove non-targeted compounds:**

146 To convert soluble alginate (Na^+-Alg and Mg^{2+}-Alg) into Ca^{2+}-Alg gel in the sediment suspension, 100 mL of 10 % (w/v)

147 calcium chloride ($CaCl_2$, Wako, purity ≥ 95 %) solution was added to the sample and stirred for 5 min. Subsequently,

148 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

149 temperature (25 °C). After the reaction, the resulting approximately 900 mL suspension was divided into eight 45 mL

150 centrifuge tubes and centrifuged (3500 rpm, 10 min, 2380 × g). The supernatant from each tube was carefully removed, and

151 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

152 centrifugation to remove the acidified supernatant. This acid treatment and the subsequent removal of the supernatant served

153 to eliminate polyvalent cations (e.g., Ca^{2+} and Mg^{2+}), acid-soluble phenolic compounds, and easily degradable polysaccharides

154 such as fucoidans from the sediment sample (Bertagnolli et al., 2014). During this process, a portion of the Ca^{2+}-Alg was

155 presumed to have undergone ion exchange with H^+, forming insoluble H^+-Alg (McHugh et al., 2001). Following this step,

156 alginate in the sediment sample was presumed to exist as a mixture of gelled forms (Ca^{2+}-Alg and Fe^{3+}-Alg) and protonated

157 alginate (H^+-Alg) in a gelatinous form. Pectin was presumed to have been removed with the supernatant, because

158 depolymerized pectin is generally considered unable to form stable gel networks through Ca^{2+} crosslinking.

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

160 Most of the sediment containing alginate gels in each tube was transferred to a single beaker using a spoon. To recover any

161 remaining precipitate in tubes, all eight tubes were rinsed with 500 mL of 1.0 % (w/v) sodium bicarbonate ($NaHCO_3$, Wako,

162 purity ≥ 99.5 %) solution, and the rinse solution was collected into a beaker. The resulting suspension in the beaker was heated

163 to 60 °C with continuous stirring for 1.5 h, followed by reaction overnight at room temperature to convert the alginate gel to

164 Na^+-Alg. Following this step, alginate in the suspension sample was presumed to exist primarily as soluble Na^+-Alg; however,

165 if Fe^{3+}-Alg was not soluble under the alkaline conditions (pH 8.0) of the $NaHCO_3$ solution, it may have remained in a gel form,

166 as described in Sect. 3.2.1.

167 **Step 4 Gelation of Na^+-Alg into Ca^{2+}-Alg:**

168   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

169   supernatant was collected into a beaker. Subsequently, 100 mL of 30 % (w/v) CaCl$_2$ solution was added to the supernatant,

170   and the mixture was kept overnight at room temperature to convert soluble Na$^+$-Alg to Ca^{2+}-Alg gel. Following centrifugation,

171   the supernatant was removed, and the resulting precipitate was retained in the tubes. Then, an excess amount of Ca^{2+} was

172   added to completely react with the CO$_3^{2-}$ ions derived from the previously added NaHCO$_3$ solution, thereby ensuring efficient

173   ion exchange between Na$^+$-Alg and Ca^{2+}. After this step, alginate in the precipitate sample was presumed to exist as Ca^{2+}-Alg

174   (gel form).

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

176   Twenty milliliters of 0.10 mol L^{-1} HCl was added to each centrifuge tube containing the precipitates. The tubes were manually

177   shaken to ensure thorough mixing, followed by centrifugation to remove the acidified supernatant. This acid-washing treatment

178   was repeated until oligosaccharides were no longer detected in the supernatant. To verify the presence of oligosaccharides, the

179   phenol-sulfuric acid colorimetric method was employed: 1.0 mL of the supernatant was transferred to a centrifuge tube, to

180   which 1.0 mL of 5.0 % (w/v) phenol (Wako, purity ≥ 99 %) solution was added and mixed. Subsequently, 5.0 mL of H$_2$SO$_4$

181   was quickly added and mixed thoroughly. After 10 min, the appearance of a yellow color in the solution, indicating the presence

182   of oligosaccharides, was monitored. If color was observed, an additional round of acid-washing treatment was performed.

183   When the solution remained colorless, confirming the absence of detectable oligosaccharides, the acid-washing treatment was

184   terminated. The precipitates from each centrifuge tube were then collected into a single beaker using a spoon. After this HCl

185   treatment, alginate in the precipitate sample was presumed to exist as a mixture of Ca^{2+}-Alg (gel form) and H$^+$-Alg (gelatinous

186   form).

187   **Step 6 Collection of Na$^+$-Alg:**

188   One hundred milliliters of 1.0 % (w/v) NaHCO$_3$ solution was added to a beaker containing the precipitates. The suspension

189   was then heated to 60 °C with continuous stirring for 1.5 h, followed by reaction overnight at room temperature to convert

190   Ca^{2+}-Alg to Na$^+$-Alg. After centrifugation, the supernatant was collected into a clean beaker. Following this step, alginate in

191   the solution sample was expected to exist as soluble Na$^+$-Alg. This extract solution appeared dark brown, likely due to the

192   presence of residual phenolic compounds (McHugh, 1987).

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

194   To remove non-targeted compounds soluble in organic solvents, such as pigmentation, fats, and phenolic compounds, 400 mL

195   of ethanol (Wako, purity: 99.5 %) was added to the beaker containing the extract solution (Saji et al., 2022; Trica et al., 2019).

196   The ethanol–water mixture was kept overnight at room temperature to precipitate alginate, because alginate is insoluble in

197   ethanol (Gomez et al., 2009). The resulting precipitate was collected by filtration using a polycarbonate membrane filter

198   (Nuclepore, Whatman, Maidstone, UK; pore size: 10 μm, diameter: 47 mm). The filter with the retained precipitate was

199   transferred to a beaker and air-dried overnight at room temperature. Subsequently, 100 mL of 1.0 % (w/v) NaHCO$_3$ solution

200   was added to the beaker containing the dried filter, and the mixture was stirred for 1.0 h. The solution was then kept overnight

201   at room temperature to ensure complete dissolution of Na^+-Alg. After centrifugation, the supernatant was recovered; however,

202   it exhibited a slight brown coloration. To reduce the coloration and ensure compatibility with colorimetric analysis, the

203   supernatant was diluted 20-fold with Milli-Q water and analyzed colorimetrically as described in Sect. 2.2. However, if trace

204   amounts of Na^+-Alg are soluble in ethanol, significant loss may occur during the extraction of trace levels of Na^+-Alg from

205   ocean sediment, as described in Sect. 3.2.2.

2.4 Collection of phytoplankton aggregates

207   In the marine environment, phytoplankton are the dominant primary producers, and thus a substantial proportion of the organic

208   matter deposited on the seafloor is presumed to originate from them. Phytoplankton are also known to produce uronic acids,

209   including glucuronic acid derived from structural polysaccharides and galacturonic acid derived from pectin, both of which

210   are constituents of the cell wall (Bergamaschi et al., 1999; Gügi et al., 2015; Domozych et al., 2007). To ensure the accuracy

211   of the modified alginate extraction protocol described in Sect. 3.2.3, it was necessary to confirm that polyuronic acids other

212   than alginate are not detected from phytoplankton aggregate samples by the modified extraction protocol.

213   Phytoplankton aggregates were collected from the water column in Funka Bay (Hokkaido, Japan, Fig. 1) during the peak of

214   the spring phytoplankton bloom in March of 2018, 2019, and 2021. Sampling was conducted using a plankton net with a mouth

215   area of 707 cm^2 (mesh size: 300 μm; mouth diameter: 30 cm) towed from near the seafloor (87 m depth) to the surface. During

216   the bloom, the chlorophyll-a concentrations in the water reached approximately 20 μg L^{-1}. It has been reported that

217   approximately half of the annual primary production in Funka Bay occurs during the spring bloom, with a substantial portion

218   of diatom aggregates sinking and being deposited on the seafloor (Kudo et al., 2000). We assumed that the amount of

219   phytoplankton aggregates collected by the plankton net corresponded to the sedimented amount of polyuronic acids derived

220   from phytoplankton on the seafloor surface within the same area (30 cm diameter). The collected phytoplankton samples ($n =$

221   3) were processed using the modified extraction protocol (described in Sect. 3.2.3) and analyzed colorimetrically (as described

222   in Sect. 2.2) to evaluate the detectability of polyuronic acids derived from the phytoplankton samples.

2.5 Verification of alginate assisted with alginate-degrading enzyme

224   Colorimetric methods estimate alginate content by quantifying uronic acids; however, these methods may also detect uronic

225   acids derived from sources other than alginate, potentially leading to overestimation. To verify that the extracted material from

226   the ocean sediment was alginate, we employed an alginate lyase to confirm its presence through enzymatic degradation. The

227   alginate lyase used in this study was HULK (NIPPON GENE, Tokyo, Japan), which is the most effective enzyme for formation

228   of oligosaccharide from only alginate polymers.

229   HULK most preferably degrades MM blocks, by splitting glycosyl linkages of the alginate chain via the β-elimination

230   mechanism (Inoue et al., 2014). When alginate is present in the extract solution, the enzymatic reaction catalyzed by HULK

231   proceeds to degrade the alginate polymer into di- and trisaccharides. The addition of Ca^{2+} to di- and trisaccharides derived

232   from alginate does not induce gelation, because these low-molecular-weight fragments lack the chain length required for

233   effective crosslinking with Ca^{2+}. Therefore, if the extract solution was treated with HULK and subsequently subjected to Ca^{2+}

234   addition, a significant reduction in calcium-induced precipitation would indicate that the original extract had contained an

235   amount of alginate.

236   The analytical sample was 75 mL of the solution extracted from 1.8 kg of sediment collected off Muroran (Fig. 1), prepared

237   by using the modified extraction protocol (described in Sect. 3.2.3).

238   The extract solution appeared dark brown, similar in color to algal extracts rich in polyphenols as reported by

239   Andriamanantoanina and Rinaudo (2010), suggesting the possible presence of polyphenols in the sample. It has been reported

240   that polyphenols can form complexes with carbohydrates such as alginates and proteins, thereby inhibiting enzymatic activity,

241   and that the inhibition of alginate degradation by alginate lyase is correlated with the concentration of soluble polyphenols

242   (Moen et al., 1997). Therefore, it is essential to remove polyphenols from the sample prior to enzymatic degradation.

2.5.1 Bleaching treatment

244   Bleaching treatment has been reported to effectively remove pigments from alginate extracts derived from dark brown algae,

245   which are presumed to be rich in polyphenols, although direct quantification was not performed in that study

246   (Andriamanantoanina and Rinaudo, 2010). Therefore, in the present study, bleaching treatment was applied to reduce the

247   polyphenol content in the extract solution.

248   Three hundred and seventy-five milliliters of 2.4 % (v/v) sodium hypochlorite (NaClO, Wako) solution was added to 75 mL

249   of extract solution in a beaker and stirred for 20 min. Subsequently, 188 mL of Milli-Q water was added, followed by

250   neutralization with 0.05 mol L^{-1} HCl. Then, 20 mL of 30 % (w/v) CaCl$_2$ was added, and the mixture was kept overnight at

251   room temperature to convert soluble Na$^+$-Alg to Ca^{2+}-Alg gel. The following day, the precipitate was collected by

252   centrifugation and transferred to a clean beaker. Forty milliliters of 1.0 % (w/v) NaHCO$_3$ solution was added to the precipitate,

253   and the suspension was heated to 60 °C with continuous stirring for 1.5 h, followed by reaction overnight at room temperature

254   to convert Ca^{2+}-Alg to Na$^+$-Alg. Finally, the supernatant was collected by centrifugation. To reduce the coloration of the extract

255   solution as much as possible, this bleaching process was repeated six times with appropriate adjustments to the reagent

256   quantities (Table 1). After the final bleaching step, the volume of the sample was reduced to 15 mL. The same procedure was

257   also applied to 75 mL of 10 µg mL^{-1} Na$^+$-Alg solution.

2.5.2 Enzymatic degradation of alginate

259   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

260   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 %)

261   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

262   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

263   (HULK) were sequentially added to a centrifuge tube with gentle mixing after each addition. This enzymatic degradation

264 experiment was performed in triplicate using the same sample ($n = 3$). In parallel, a control experiment was performed without

265 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

266 bleached extract solution, and 0.40 mL of Milli-Q water instead of alginate lyase were sequentially added to a centrifuge tube

267 with gentle mixing after each addition. The control experiment was also conducted in triplicate ($n = 3$).

268   The same enzymatic experiment was performed on the bleached Na^+-Alg standard solution ($n = 1$). In addition, a blank sample

269 ($n = 1$) was prepared as a control using water in place of the extract solution: specifically, 5.0 mL of Milli-Q water, 0.80 mL

270 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

271 tube with gentle mixing. The mixtures were centrifuged (3500 rpm, 1 min, 2380 × g) to remove residual water from the inner

272 wall surface and subsequently incubated in an incubator (SLI-220, EYELA, Tokyo, Japan) at 30 °C for one week. After

273 incubation, the pH of each mixture was adjusted to 4.0 using 1.0 mol L^{-1} HCl. Subsequently, 10 mL of 30 % (w/v) $CaCl_2$ was

274 added, and the mixtures were kept overnight at room temperature to convert Na^+-Alg remaining in the mixture to Ca^{2+}-Alg.

275   On the following day, the resulting precipitates were collected by filtration using polycarbonate membrane filters (Nuclepore,

276 Whatman; pore size 0.40 μm, diameter 47 mm). At this stage, the oligomerized alginate produced by alginate lyase was

277 expected to remain in the filtrate, because it does not form crosslinks in the presence of Ca^{2+}. Each filter containing the

278 precipitate was transferred into a separate tube, to which 5.0 mL of 2.0 % (w/v) $NaHCO_3$ solution was added. The tubes were

279 then incubated in a 60 °C water bath for 1.5 h with gentle shaking every 10 min, followed by reaction overnight at room

280 temperature to convert Ca^{2+}-Alg to Na^+-Alg. The supernatants were collected by centrifugation (3500 rpm, 10 min, 2380 × g),

281 and analyzed colorimetrically as described in Sect. 2.2.

282

283 **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)	CaCl₂ aq (mL)	NaHCO₃ 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

284 a: NaClO concentration was 3.6 % for this experimental run only; the concentration in all other runs was 2.4 %.

285

3 Results and discussion

3.1 Spike recovery of alginate using the conventional food chemistry method

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

Table 2 Spike recovery of added Na^+-Alg from ocean sediment by using the food extraction method[b]

Sample	Absorbance	Yield (mg per 300 g)	Spike recovery[a] (%)
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

a: Spike recovery = Yield (mg per 200 mg)
b: Conventional extraction method used in food chemistry

3.2 Modification of extraction treatment

3.2.1 Dissolve Fe^{3+}-Alg present in the sediment sample

High concentrations of iron ions are commonly present in the pore water of ocean sediments (Klinkhammer, 1980). Fe^{3+} interacts with alginate to form a stable gel, whereas Fe^{2+} exhibits poor cross-linking ability with polysaccharides and thus does not effectively convert alginate from its soluble form to a gel (Roquero et al., 2022). Fe^{3+}-Alg gel has been reported to exhibit higher stability relative to gels formed with other divalent or trivalent metal ions such as Ca^{2+}, Mg^{2+}, and Al^{3+} (Menakbi et al., 2016). If Fe^{3+}-Alg, formed via ion exchange between alginate and Fe^{3+}, is present in a sediment sample, the gel may not dissolve in the $NaHCO_3$ solution and could remain in the sediment even after alkaline treatment (Step 3), leading to reduced recovery. To address this issue, we investigated the optimal conditions for the simultaneous dissolution of both Fe^{3+}-Alg and Ca^{2+}-Alg gels, which are presumed to be the predominant forms of alginate present prior to the alkaline treatment. Disodium dihydrogen ethylenediamine tetraacetic acid dihydrate (EDTA-2Na) is a potent chelator that forms more stable complexes with Fe^{3+} than the binding affinity of Fe^{3+} to alginate (Berner and Hood, 1983). Because the stability of EDTA-metal complexes depends on pH (Ueno, 1959), we investigated the dissolution behavior of Fe^{3+}-Alg and Ca^{2+}-Alg in EDTA-2Na solutions adjusted to pH 4.0 and 5.0, and compared the results with those obtained using $NaHCO_3$ solution at pH 8.0.

Fe^{3+}-Alg gel was prepared by dropping 20 mL of 1.0 % (w/v) Na^+-Alg solution into 20 mL of 10 % (w/v) iron (III) chloride hexahydrate ($FeCl_3$, Wako, purity ≥ 99 %) solution in a beaker. To ensure complete reaction between Na^+-Alg and Fe^{3+} ions, the resulting Fe^{3+}-Alg/Na^+-Alg suspension was repeatedly filtered through a 100 μm stainless steel mesh using a silicone spatula. The reddish Fe^{3+}-Alg gel retained on the mesh was collected and rinsed three times with Milli-Q water. For comparison,

315   Ca^{2+}-Alg gel was similarly prepared by dropping 20 mL of 1.0 % (w/v) Na$^+$-Alg solution into 20 mL of 10 % (w/v) CaCl$_2$

316   solution. Each gel type (Fe^{3+}-Alg and Ca^{2+}-Alg) was divided into two portions. One half of each was added to 500 mL of 1.0 %

317   (w/v) NaHCO$_3$ solution and heated to 60 °C with continuous stirring for 1.5 h. The other halves were added to 500 mL of

318   8.0 % (w/v) EDTA-2Na (Kanto Chemical, Tokyo, Japan, purity > 99.5 %) solution, which had an initial pH of 4.0, and stirred

319   for 1.5 h. These suspensions were then kept overnight at room temperature. The presence or absence of remaining gel was

320   assessed visually. Subsequently, the pH of the EDTA-2Na solution was adjusted to 5.0 by adding 3.0 mol L^{-1} NaOH solution.

321   After stirring for an additional 1.5 h, the mixtures were again kept overnight at room temperature, and the presence of gel was

322   assessed visually.

323   Most of the Fe^{3+}-Alg did not dissolve in NaHCO$_3$ (pH 8.0) solution, but it did dissolve completely in EDTA-2Na solution at

324   pH 4.0 and pH 5.0 (Table 3). Ca^{2+}-Alg, in contrast, was soluble in both NaHCO$_3$ (pH 8.0) and EDTA-2Na (pH 5.0) solutions,

325   but it remained insoluble in EDTA-2Na at pH 4.0. Based on these observations, EDTA-2Na solution at pH 5.0 was selected

326   as the optimal condition for the simultaneous dissolution of Fe^{3+}-Alg and Ca^{2+}-Alg in ocean sediment samples.

327

328   **Table 3 Results of dissolution tests on Fe^{3+}-Alg and Ca^{2+}-Alg in NaHCO$_3$ 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

329

330

3.2.2 Omit ethanol precipitation in the final purification step

332   In the field of food chemistry, Na$^+$-Alg is generally considered to be insoluble in ethanol–water mixtures; therefore,

333   precipitation of Na$^+$-Alg in a 4:1 (v/v) ethanol–water solution is commonly employed as the final step in extraction and

334   purification. However, we were concerned that trace amounts of Na$^+$-Alg might dissolve in a 4:1 (v/v) ethanol–water solution.

335   If such minor solubility exists, it could result in significant loss during the extraction of trace levels of Na$^+$-Alg from ocean

336   sediment.

337   To evaluate the dissolution behavior, we prepared two Na$^+$-Alg solutions (0.05 % and 0.20 %, w/v; 100 mL each) and added

338   400 mL of ethanol (99.5 %) to each. The mixtures were kept overnight at room temperature to allow precipitation. Afterward,

339   each mixture was filtered through a polycarbonate membrane filter (Nuclepore, Whatman; pore size: 0.22 μm, diameter: 47

340   mm). The filtrates were collected and evaporated to complete dryness. The resulting residues were re-dissolved in 20 mL of

341   Milli-Q water. Subsequently, 0.50 mL of each re-dissolved sample was transferred to a separate tube and diluted 40-fold with

342   Milli-Q water. The concentration of Na$^+$-Alg in each diluted sample was then determined using the *m*-hydroxydiphenyl

343   colorimetric method.

344   We observed that the 0.20 % Na$^+$-Alg solution was partially soluble in ethanol, with approximately 66 % dissolution, whereas

345   the 0.05 % Na$^+$-Alg solution was completely dissolved (Table 4). These results indicate that trace amounts of alginate are

346   soluble in ethanol. Consequently, a significant loss of alginate could occur during ethanol precipitation, particularly when

347   extracting trace levels of alginate from ocean sediment. Therefore, the ethanol precipitation process was considered unsuitable

348   for the extraction of alginate from sediment samples.

349

350   **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
0.05 %	0.050	0.057[a]	100 %
0.20 %	0.200	0.132	66 %

351   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
352   precision (±15 %).

353

354   **3.2.3 Modified extraction protocol**

355   We modified two key aspects of the alginate extraction protocol. First, an EDTA-2Na treatment step was introduced to

356   dissolve Fe^{3+}-Alg and Ca^{2+}-Alg gels into the liquid phase. Second, the ethanol precipitation step was omitted from the final

357   purification process to avoid significant loss of alginate. The modified extraction protocol, in which Steps 1, 2, 4, and 5 remain

358   identical to those described in the Methods section, is presented below. A complete overview of the updated protocol for

359   alginate extraction from ocean sediment is provided in supplementary material (Fig. S1).

360   We prepared nine sediment samples with a wet weight of 300 g using muddy sediment rich in organic material collected from

361   Funka Bay (Fig. 1) in March 2024. The test samples ($n = 3 \times 3$) were treated by the modified alginate extraction method as

362   follows (Steps 1–7).

363   **Preparation of test sediment samples:**

364   To prepare the test samples, 500 mL of Milli-Q water and 20 mL of Na$^+$-Alg solutions at concentrations of 1.0 %, 0.50 %, and

365   0.25 % (w/v)—containing 200 mg, 100 mg, and 50 mg of Na$^+$-Alg, respectively—were added to 300 g of sediment sample (n

366   $= 3$) in separate beakers, followed by stirring for 5 min. These samples were labeled as "sediment + Na$^+$-Alg 200 mg,"

367   "sediment + Na$^+$-Alg 100 mg," and "sediment + Na$^+$-Alg 50 mg," respectively. The resulting suspensions were stored

368   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,

369   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

370   and Mg^{2+}-Alg).

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

372   To prepare 8.0 % (w/v) EDTA-2Na solution, 40 g of EDTA-2Na was dissolved in 500 mL of Milli-Q water. The pH of the

373   resulting solution was 4.0 without any adjustment. Most of the sediment containing alginate gel from each tube was transferred

374   into a single beaker using a spoon. To recover any remaining precipitate in tubes, all eight tubes were rinsed with 500 mL of

375   8.0 % EDTA-2Na solution (pH 4.0), and the rinse solution was collected into a beaker. The resulting suspension in the beaker

376 was stirred for 1.5 h, followed by reaction overnight at room temperature to convert Fe^{3+}-Alg to Na^+-Alg via chelation.

377 Subsequently, the pH of the suspension was adjusted to 5.0 by adding 3.0 mol L^{-1} NaOH solution, followed by stirring for

378 another 1.5 h and reaction overnight at room temperature to convert Ca^{2+}-Alg into Na^+-Alg through ion exchange. Following

379 this step, alginate in the suspension sample was presumed to exist primarily as soluble Na^+-Alg.

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

381 An appropriate volume (20–100 mL) of 1.0 % (w/v) $NaHCO_3$ solution, adjusted based on the estimated amount of alginate,

382 was added to a beaker containing the precipitates to keep the alginate concentration within the range of the calibration curve

383 (Fig. 2). For instance, 100 mL of 1.0 % $NaHCO_3$ solution was added to the sample that had been supplemented with alginate

384 during the preparation step, because it was presumed to contain a large amount of Ca^{2+}-Alg. Conversely, for the sample without

385 added alginate, which was presumed to contain a trace amount of Ca^{2+}-Alg, only 20 mL of 1.0 % $NaHCO_3$ solution was used.

386 The suspension was then heated to 60 °C with continuous stirring for 1.5 h, followed by reaction overnight at room temperature

387 to convert Ca^{2+}-Alg to Na^+-Alg. After centrifugation, the supernatant was collected into a clean bottle. Following this step,

388 alginate in the solution sample was expected to exist as soluble Na^+-Alg.

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

390 The extract solution appeared dark brown, likely due to the presence of residual phenolic compounds (McHugh, 1987). To

391 reduce the coloration and ensure compatibility with colorimetric analysis, the extract solution was diluted 20–100-fold with

392 Milli-Q water and analyzed colorimetrically as described in Sect. 2.2.

393 **3.2.4 Collection efficiency of alginate using the modified protocol**

394 Table 5 shows the yield of alginate extracted from the sediment sample and the corresponding spike recovery based on the

395 modified extraction protocol. We achieved higher spike recoveries (64.7–82.6 %) by using the modified extraction protocol

396 than by the conventional protocol (recovery 38.7 %; Table 2). It should be noted that these efficiencies reflect the recovery of

397 alginate naturally present in the sediment samples; therefore, the reported values may be slightly overestimated.

398

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

Sample	Absorbance	Yield (mg per 300 g)	Spike recovery[b] (%)
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

	0.080	33.63	67.3
Sediment + Na$^+$-Alg (50 mg)	0.090	37.33	74.7
	0.092	38.07	76.1
			average 72.7

a: The modified protocol includes Fe^{3+}-Alg dissolution and does not involve ethanol precipitation.

b: Spike recovery = Yield (mg per 200 mg, 100 mg, or 50 mg)

3.3 Interference by polyuronic acid derived from phytoplankton

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

Table 6 Yield of uronic acid from plankton net samples

Plankton net sample	Chlorophyll-a (g per sample)	Modified extraction	
		Absorbance	Yield (mg per sample)[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

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

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

b: These absorbances exceed the lower limit (absorbance = 0.0037) described in Sect. 2.2.

3.4 Reduction in amount of alginate after enzymatic degradation

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

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 mL^{-1} after enzymatic treatment (Table 7). A similar reduction was observed for the Na$^+$-Alg standard solution, which decreased 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 extract and the standard solutions was effectively degraded by the enzyme, thereby supporting the presence of alginate in the extracted material.

425 However, the extract solution retained relatively high Na$^+$-Alg concentrations (11.81–12.37 µg mL^{-1}) even after enzymatic

426 treatment. This result suggests that the bleaching treatment was insufficient to completely remove polyphenols, which may

427 have interfered with alginate lyase activity. In addition, the absorbance of the water sample treated with alginate lyase was

428 0.004, indicating that residual enzyme in the measurement sample contributed slightly to the colorimetric reading; however,

429 this effect was considered negligible. Taken together, these findings indicate that the organic matter extracted from ocean

430 sediments using the modified protocol contained alginate, accounting for at least one-third of the uronic acids detected.

431

432 **Table 7 Na$^+$-Alg concentrations of extract solution, Na$^+$-Alg standard solution, and Milli-Q water (blank), both with and without**
433 **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	-

434

435

436 **3.5 Amount of alginate in the surface of ocean sediment in a coastal subarctic area**

437 The modified extraction protocol was applied to the ocean sediment samples (300 g) collected from the surface 0–1 cm depth

438 in the coastal area around southern Hokkaido, Japan (Fig. 1). Abundant alginate was detected in all extracted samples, with

439 values of 0.55–6.67 mg per 300 g (alginate/wet weight of sediment). In Funka Bay, alginate concentrations were 0.55–2.34

440 mg per 300 g, corresponding to 6.11–26.0 mg m^{-2} (alginate/surface sediment area) during the sampling period from August to

441 December 2024. In Hakodate Bay and off Cape Esan, concentrations were 3.51–3.72 mg per 300 g (39.0–41.3 mg m^{-2}), and

442 1.06–1.32 mg per 300 g (11.8–14.7 mg m^{-2}), respectively, during June 2024. Off Muroran, concentrations were 5.25–6.67 mg

443 per 300 g (corresponding to 58.3–74.1 mg m^{-2}) during February 2025. To estimate the future flux of alginate from brown algae

444 to the ocean floor, it is essential to determine both the degradation rate of alginate in seawater and sediments, as well as its

445 standing stock.

446

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

Sample	Absorbance	Yield (mg per 300 g)[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

448 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-
449 fold for those from off Muroran.
450 b: Due to the amount of sediment collected, the number of samples was two ($n = 2$).

451

4 Conclusions

453 Recently, the carbon sequestration potential of macroalgae has received increasing attention as a means of mitigating
454 atmospheric CO_2 levels. In this study, we developed an extraction protocol for alginate (a polysaccharide primarily derived
455 from brown algae) in marine sediments, based on a conventional method used in food chemistry, and evaluated its applicability
456 for quantitative analysis. Spike recovery tests with Na^+-Alg (200, 100, and 50 mg) demonstrated substantially higher recoveries
457 (64.7–82.6 %) by using the modified protocol than with the conventional method (38.7 %). To assess potential interference
458 from non-alginate polyuronic acids, such as those derived from phytoplankton, phytoplankton aggregates were subjected to
459 the modified protocol. Most of the interference was effectively eliminated, and the residual contribution was minor (23 %)
460 relative to the estimated alginate content in marine sediments at the same site. To confirm that the uronic acids in the sediment
461 extracts originated from alginate, an enzymatic degradation test using alginate lyase was performed on natural extracts. The
462 uronic acid concentration decreased by approximately one-third after enzymatic degradation, indicating that a portion of the
463 uronic acids was derived from alginate. Finally, the alginate content of marine sediments collected around southern Hokkaido,
464 Japan, was quantified using the modified protocol. Concentrations were 6.11–26.0 mg m^{-2} in Funka Bay (August–December
465 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 58.3–74.1 mg m^{-2}

466 off Muroran (February 2025). These findings provide the first quantitative assessment of alginate preserved in marine

467 sediments in this region, highlighting its potential contribution to coastal carbon sequestration. To more accurately predict the

468 flux of alginate from brown algae to the seafloor, future studies should determine the degradation rate of alginate in seawater

469 and sediments, as well as its standing stock.

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