



HOKKAIDO UNIVERSITY

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**Studies on speciation of iron and its
bioavailability for the recovery of
seaweed-bed of barren coast using a steel
slag-compost fertilizer**

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Chapter 1

General Introduction

1.1. Barren ground in coastal areas

Algae mean very simple plants, which are growing in aquatic environments with no real leaves, stems or roots. In general, algae are called “Seaweeds” traditionally, which include only macroscopic marine green, red and brown algae, but not include the phytoplankton [1]. Seaweeds are also evolutionarily quite diverse – Cyanophyta (blue green algae), Chlorophyta (green algae), Rhodophyta (red algae) and Phaeophyta (brown algae). As shown in Fig 1.1, seaweeds have important functions from ecological and economical points of view. Seaweeds are widely utilized to a variety of industrial products (e.g., alginates, agar and carragenans for emulsifier, aggregator and excipients), animal fodder and fertilizer [1,2]. In Japan, seaweeds have composed an important part of food products (Nori, Kombu and Wakame) for a long time. In recent years, nutritional value of the marine algae is attracted as food supplements for human health [3]. Moreover, the algae have also been considered as potential of bio-fuels, and pharmacological uses [1,4]. For coastal ecosystems, seaweed beds serve as habitats for fishes, invertebrates and benthos (Fig. 1.1). The seaweed beds have been recognized as important areas for the egg attachment of invertebrates and fishes, the shelter of juvenile fish and creating the high biodiversity coastal area [1,4]. Recently, the reduction of CO₂ emission is attracted, and seaweed cultivation can contribute to the fixation of carbon. According to some reports, brown macro algae forest has high potential of biomass productivity, which can be estimated to be $1 \sim 3.4 \text{ kg-C m}^{-2} \text{ year}^{-1}$. These values for macro algae are two times higher than those of tree plantation or grassland in temperate region [4-7].

As mentioned above, the preservation and cultivation of seaweed beds are

crucial important for industrial uses and protecting marine ecosystems. However, seaweeds can be easily depleted by herbivorous damage, human impact, nutritional and various environmental changes. Seaweed deforestation has been well known as a coastal environmental problem in East Asia, Canada and Australia [8-14]. The extinction of seaweed at Sydney Harbor has been first reported in 2003, due to dredging, trawling, siltation and seabed destruction [13]. Seaweed depletion is a conspicuous phenomenon in worldwide [10]. Between 1978 and 1991, the presence of seaweed beds (seagrass and *Sargassum* beds) was confirmed at 41.6 % of 201,212 ha surveyed areas among the coastal areas in Japan. However, 18,538 ha (22 % of the confirmed algae beds areas) was lost until 2006 ~ 2008 [8,15]. The major of those losses seems to be caused by land reclamation or construction, tidal flats and other human impact. For example, more than 90 % of Tokyo Bay was land-reclaimed and it leads to the losses of seaweed beds. Apart from this type deforestation, barren ground of coastal areas due to various environmental factors is also recognized as a serious coastal problem, that is, “*Isoyake*” in Japanese since the 1800s [8,12]. The barren ground in coastal area is a phenomenon where seaweed beds are decreased or extinct, and coralline algae containing calcium carbonate components cover rock surface (Fig. 1.2). Some reasons for the barren ground have been considered until today, the following four reasons are mainly expected: the (i) biological damage, (ii) insertable bottom, (iii) aquatic environmental change and (iv) oligotrophic nutrition theory [8,17,18]. The first reason means that seaweed may be heavily grazed by sea urchin, and this is conspicuous as a cause of seaweed depletion in many reports [8-10,12,14]. The second reason represent that the attachable rocks are needed to growth of seaweed. In the restoration technique based on this, it is considered that construction of artificial algal reefs (e.g., sinking special shaped concrete blocks)

can provide the places for seaweed attachment to form the alga superiority population within the short time [8,11,16,20]. A lot of researches of fisheries and phycologists have tried to create artificial seaweed beds using concrete structures since 1980 [20], and huge costs have been invested in this project in China [11]. However the aforementioned method intensively alters coastal environments and ecological systems, and have promoted without sufficient monitoring and scientific evidences. The third reason means habitat of seaweed is physiologically changed by unsuitable conditions, such as the lost of tidal flats, the decreasing of light intensity by low transparency in coastal seawater and the increase of seawater temperature by global warming [8,12,19]. In addition, the lack of essential micronutrients, especially Fe, may bring about poverty of seaweeds and/or condition in which seaweeds cannot have a stage of producing gametes [8,17,18]. Matsunaga et al. [17] reported that the total iron concentrations in the barren coast for the Japan Sea were ultra trace levels, less than 2 nM. Based on these data, projects for supplying Fe to the barren coasts had carried out using iron cage or steel slag. These projects resulted in increasing iron concentration up to 10 ~ 50 μ M and seaweed growth (e.g., the increased pigment of seaweed at the areas of Fe supply [17], the restoration of seaweed beds in a barren coastal area at Erimo in Hokkaido, Japan [18]). It is presumably that there is a variety of reasons for barren ground at each coastal area. Thus, a variety of techniques for restoration of seaweed beds have been attempted based on the hypothesis in the various coastal areas of barren ground in Japan. Matsunaga et al. [18] pointed that the lack of iron in barren coastal areas is due to suburban developments, that is, constructions of dams and shore protections can suppress the iron input from river to coast. However, no one has fundamentally understood why iron is needed to restore the seaweed beds in barren coast. Therefore

more scientific investigations are required from biological point, as well as physical and chemical point of view, in terms of relations between iron and growth of seaweeds.

1.2. Role of iron for the growth of brown macro algae

According to the Liebig's law of minimum, the growth of a plant is dependent on the limited nutrients [1]. Nitrogen, phosphorus and trace metals as essential nutrients in seawater might limit algal growth [1]. Especially, iron has been attracted to the growth of marine algae by marine chemists and phycologists. Many studies on iron bioavailability for marine micro algae, such as phytoplankton, have been reported (e.g., [21-23]). As for the lower concentration of bioavailable iron, it is to lower quantity of algal biomass with the high-nitrate and low-chlorophyll (HNLC) contents. Iron enrichment with HNLC areas, shown in the represented as IronEx [45,46], SOIREE [47], SOFeX [48] and SEEDS [49,50] projects, confirmed the validity of hypothesis for the limitation of iron. For example, the SEEDS-II project observed a significant large increase in phytoplankton, the decreased macronutrients and dissolved carbon dioxide. These issues suggest that dissolved iron is related to the micro algal biomass and/or bloom in HNLC ocean area [50]. As aforementioned in section 1.1, the deficiency of Fe in a coastal area leads to barren ground. For the case of seaweed, iron is well known as an essential nutrient for growth, synthesis of pigments and activated groups in porphyrin and enzymes in algae [1]. Viaroli et al. [24] showed that the *Ulva* (Chlorophyta) growth and bloom are especially depended on bioavailable N and Fe. Suzuki et al [25]. showed that the growth of juvenile sporophytes for *Laminaria* and *Undaria* (Pheophyta) are enhanced significantly under the Fe-rich conditions but not for *Lithophyllum*

(Rhodophyta). In addition, iron plays an important role in the life history of brown macro algae. As shown in Fig. 1.3, *Laminaria* algae have a unique life history. The sporophytes cultivate the sori and release the zoospores, and the zoospores develop the gametophytes (male and female). After maturation of the gametophytes, gametes are released, fertilized, and then grow into sporophytes. In this cycle, Motomura and Sakai [26,27] proved that iron is required on the reproductive growth (i.e., gametogenesis) stage of *Laminaria japonica* and *angustata* (Phaeophyta). The iron-regulation on oogenesis is also confirmed for other brown macro algae (*Undaria pinnatifida*) [28]. In addition, light strength and water temperature also influences on the gametogenesis [29]. In the iron-free conditions, the gametogenesis is not induced and they continued vegetative growth only (Fig. 1.3). Therefore, when lacking iron in seawater, sporophytes cannot be formed, and this leads to seaweed depletion. To better understand the relations between iron and seaweed depletion, speciation and bioavailability of iron should be addressed by considering the conditions in seawater.

1.3. Dissolved organic matter in aquatic environments

According to data summarized by Tipping [30], the majority of carbon is fixed as sedimentary rocks on the earth (ca. 8.0×10^7 Gt-C). The remaining carbons (ca. 4.0×10^4 Gt-C) are present in biosphere or shallow substance, among which 90 % of it is existing as carbonate dissolved in seawater, and approximately 9 % (ca. 3.8×10^3 Gt-C) in organic form. Seawater (ca. 1.7×10^3 Gt-OC) and soil (ca. 1.5×10^3 Gt-OC) are can serve as major pools. For organic carbon (OCs), the natural organic matter (NOM) can be roughly fractionated to humic (HSs) and non-humic substances (non-HSs). HSs are

classified to following three fractions: humin, humic (HAs) and fulvic acids (FAs), which are based on the water solubility in acid and alkali. Humin is defined as an insoluble fraction in both acid and alkaline aqueous, HA for soluble fraction in alkaline aqueous but not in acid, and FA for soluble fraction in both alkaline and acid aqueous. The humin is an organic matter that HA absorb to mineral surface strongly, and have structural features for highly condensed insoluble humic matter, fungal melanins and paraffinic substances [30,31]. HAs and humin are accumulated to solid phase in soils, and FAs are mostly distributed in aquatic phase in natural environment. In addition, dissolved organic matter (DOM) or dissolved organic carbon (DOC) is also widely used as nomenclature in environmental science. HAs, FAs and/or DOM have been regarded as heterogeneous mixture of amorphous polymeric organic compounds. Because a variety of polar functional groups, such as carboxylic acids, phenolic hydroxyl groups and N or S-containing functional groups, are distributed in their structures, HAs, FAs and DOM have binding capabilities to various metal cations [30]. Such the functions of HSs and DOM play important roles in determining the water solubility, mobility, speciation and bioavailability of trace metals in aquatic environments.

It has been reported that the concentrations of Fe and Al in water are correlated with the concentration of DOM, suggesting that water solubility of these metal ions can be enhanced by dissolving as organic complexes [30,32]. This contributes to the transportation of Fe from lands to coastal areas *via* rivers [33]. Strictly speaking, environmental effects of the complexation are not fully understood, and this is due to multi-ligand characters [34], lability [35] and sterical or conformational complexity [36,37]. The metal complexation with HSs or DOM to trace metal ions, and their bioavailability to aquatic microorganisms are interesting topic in environmental science.

The iron complexation with DOM results in positive effects on biota in aquatic ecosystems (e.g., Chen et al. [38,39]). This may be attributed to the fact that complexation with DOM can enhance bioavailability of metal ions in aquatic environment. The Free Ion Activity Model (FIAM) proposed by Morel [40] is known to be major concept for the bioavailability of trace metal ions to microalgae. In this model, free metal species can be absorbed to algae, the presence of DOM leads to the inhibition of uptake or storage of metal ions. As explained by Aristilda et al. [41], three mechanisms for uptake of metal ions are hypothesized: (1) the metal ions are transported into the inside of cell membrane as complex forms; (2) the dissociated metal ions from metal-DOM complexes on the vicinity of cell membrane are absorbed, in which free metal ions have highly bioavailable depending on diffusion flux of the complex species; (3) the metal ions in the complexes are absorbed *via* ligand-exchange reaction with a metal binding site on the surface of cell membrane. However, mechanisms for the metal uptake to algae are controversial. In the evaluation of metal binding abilities, weak and strong binding sites have been assumed in HA, FA and DOM [30,34]. Weak binding sites are assumed as exchangeable binding with relatively weak force, e.g., electrostatic interaction and hydrogen bonding [30], and strong ligands are stable and unexchangeable sites with lower bioavailable metal species. For example, stable chelate and colloid metal species are not directly absorbed to aquatic biota, i.e., unavailable species [41]. The lability of metal-HA, FA or DOM complexes would be related to the dissociation of the complexes and the metal uptake [42].

It is demonstrated that DOM contributes to the redox cycle of Fe in aquatic environments [43,44]. In particular, Fe reduction can be accelerated by light irradiation, and this can be attributed to the ligand-to-metal charge transfer (LMCT) [43]. The

reactivity of such the reaction is dependent on the origins and structural features of HAs, FAs and DOM. Thus, metal ion species and bioavailability in aquatic environments should be discussed, based on the complexing abilities and structural features of HA, FA and DOM.

1.4. A novel technique for supplying dissolved Fe using DOM

As mentioned in previous sections, barren coastal ground is a serious problem in fisheries and coastal ecosystems. Matsunaga et al. [17,18] had pointed the Fe deficiency as one of reasons for this problem. Although the lack of dissolved Fe may be a key factor for depleting seaweed beds in barren coast (section 1.2, Fig. 1.3), supplying dissolved Fe is difficult due to their low solubility under oxic and weak alkaline conditions like coastal seawater. The limitation of solubility for Fe^{2+} and Fe^{3+} in seawater (pH 8.0 ~ 8.1) can easily be calculated by solubility products of their hydroxides ($K_{\text{sp}} = 2 \times 10^{-14} \text{ M}^2$ for $\text{Fe}(\text{OH})_2$ and $1 \times 10^{-36} \text{ M}^3$ for $\text{Fe}(\text{OH})_3$). The estimated limited concentrations of Fe^{2+} and Fe^{3+} were $2.8 \times 10^{-3} \text{ g L}^{-1}$ and $1.7 \times 10^{-18} \text{ g L}^{-1}$, respectively, suggesting that Fe^{2+} is relatively soluble in seawater. However, under oxic conditions (around $E_h = 0$) in coastal seawater, Fe^{2+} can easily be oxidized to Fe^{3+} and immediately form solid hydroxides (Fig. 1.4). In such conditions, complex species of Fe with organic matter are dissolving in aqueous solution as stable forms. Thus, the Nippon Steel & Sumitomo Metal Corporation, the University of Tokyo and other companies in Japan have attempted the restoration of seaweed beds in barren coast by supplying Fe using a fertilizer comprised of steel slag and compost as sources of iron and HSs or DOM, respectively [51]. As shown in Fig. 1.5, this approach resulted in the

increase of Fe concentration and the restoration of seaweed bed in the barren coast. However, little is known about iron species derived from the fertilizer and about their bioavailability to brown macro algae [1]. Although a variety of fundamental studies has been required and examined to elucidate the factors and indices for obtaining higher performance of fertilization, HSs in compost are mainly focused as mechanistic aspects. In addition, no approaches have been examined from phycological point of view. HSs are known to coagulate in the higher ionic strength such as seawater ($I = 0.7$) [52]. HSs are regarded as colloidal matter. According to the DLVO theory, stability of the colloids is dependent on the surface properties of particle (i.e., van der Waals force and electrostatic repulsion between the diffuse double layers). In the higher ionic strength, such as seawater with the higher concentrations of Mg^{2+} and Ca^{2+} , the large negative electrostatic field of HSs can be neutralized by counter cations and their coagulation can be observed. Actually, when seawater added to HS solutions, it was demonstrated that HA and FA extracted from bark compost were suspended immediately and coagulated within a half of day (Fig. 1.6). These results suggest that HA and FA cannot function as carriers of dissolved iron from the fertilizer to seawater. Thus, DOM derived from the fertilizer may be different from the fractions for HSs. This leads to a suggestion that fractions extracted with seawater should be addressed. In the present study, I focused on a seawater extractable organic matter (SWEOM) in the compost that may play an important role in Fe supplying. Thus, structural features and Fe-binding capabilities of SWEOM and bioavailability of Fe-SWEOM complexes to brown macro algae were investigated to elucidate functions of SWEOM relating to the Fe species and bioavailability.

The outline in this thesis is summarized as below.

Chapter 1, in this chapter, shows a general introduction of the present study. Previous technique for recovering seaweed bed and a variety of reasons for barren grounds were introduced, and problems to solve were discussed.

Chapter 2 indicates the structural features of SWEOM. The methods for the extraction and structural features of SWEOM were investigated by comparing with those for HA and FA.

Chapter 3 shows that binding abilities of HA, FA and SWEOM to ferrous iron. The binding abilities were evaluated by colorimetric titration method to calculate the thermodynamic parameters of Fe(II)-SWEOM.

Chapter 4 shows a novel analytical method for determining of complexating abilities of SWEOM with ferrous iron under seawater condition ($I = 0.7$, pH 8.0). The analytical reaction was based on a ligand-exchange reaction between Fe-SWEOM and ferrozine as a model of binding site on algal cell surface and labile species of Fe(II)-SWEOM was determined. Conditional stabilities constant and binding capacities were evaluated by complexometric titration with colorimetry.

Chapter 5 shows the bioavailability of Fe complexed with SWEOM and various chelating agents. To evaluate the bioavailability of iron(II) species the effects of iron on the maturation of gametophytes for brown macro algae (*Laminaria japonica*) were investigated.

In Chapter 6, the summary of the present study is described.

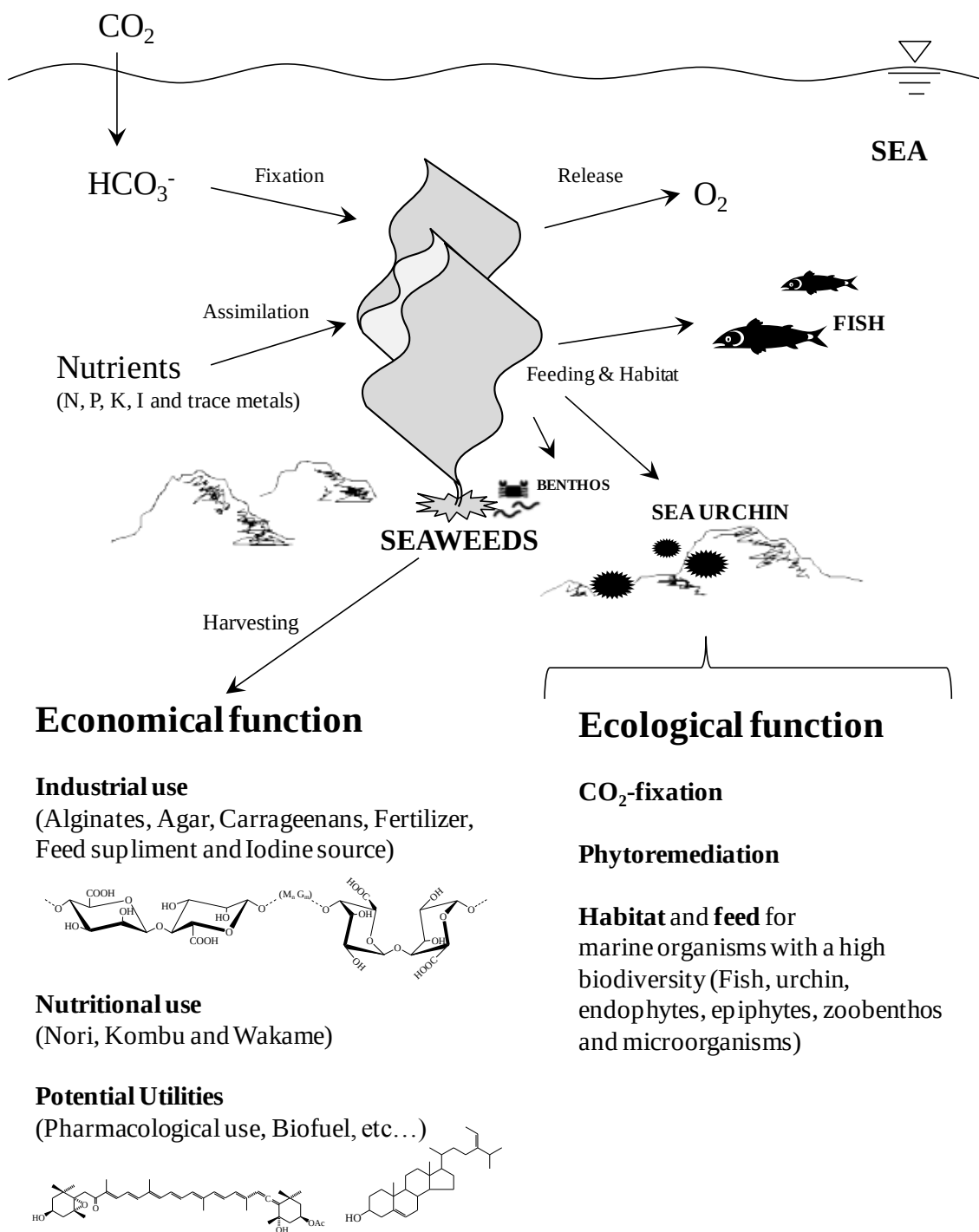


Fig. 1.1. Illustration of roles of seaweeds in economical and ecological functions.

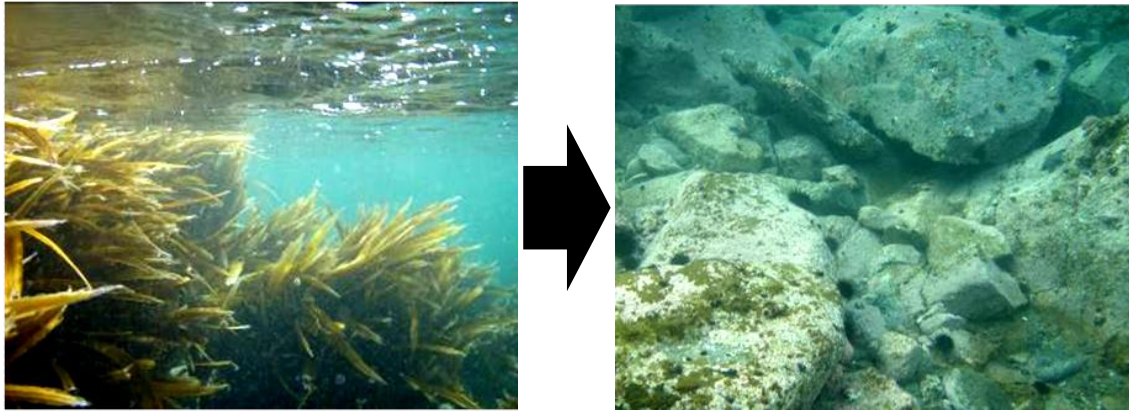


Fig. 1.2. Pictures of seaweed forest (left) and barren coastal ground (right).

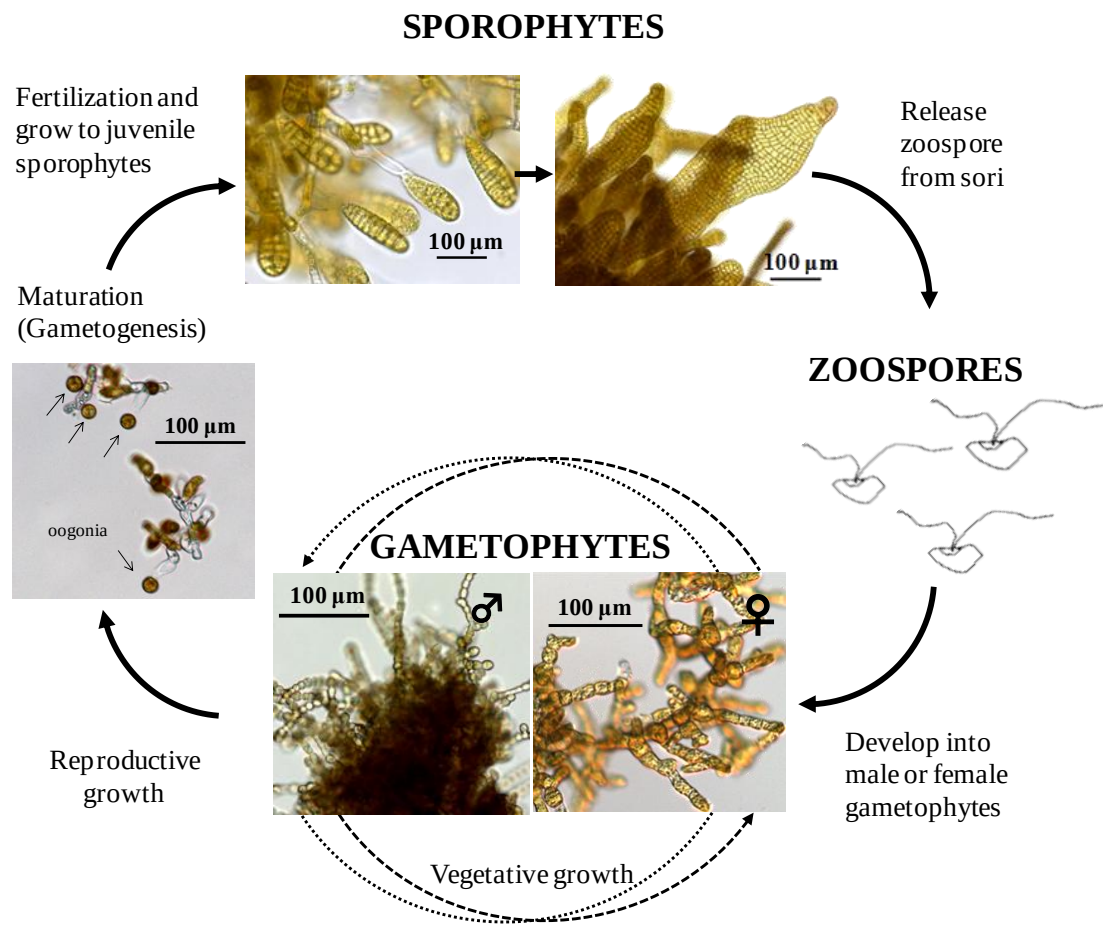


Fig. 1.3. Life history of *Laminaria* algae.

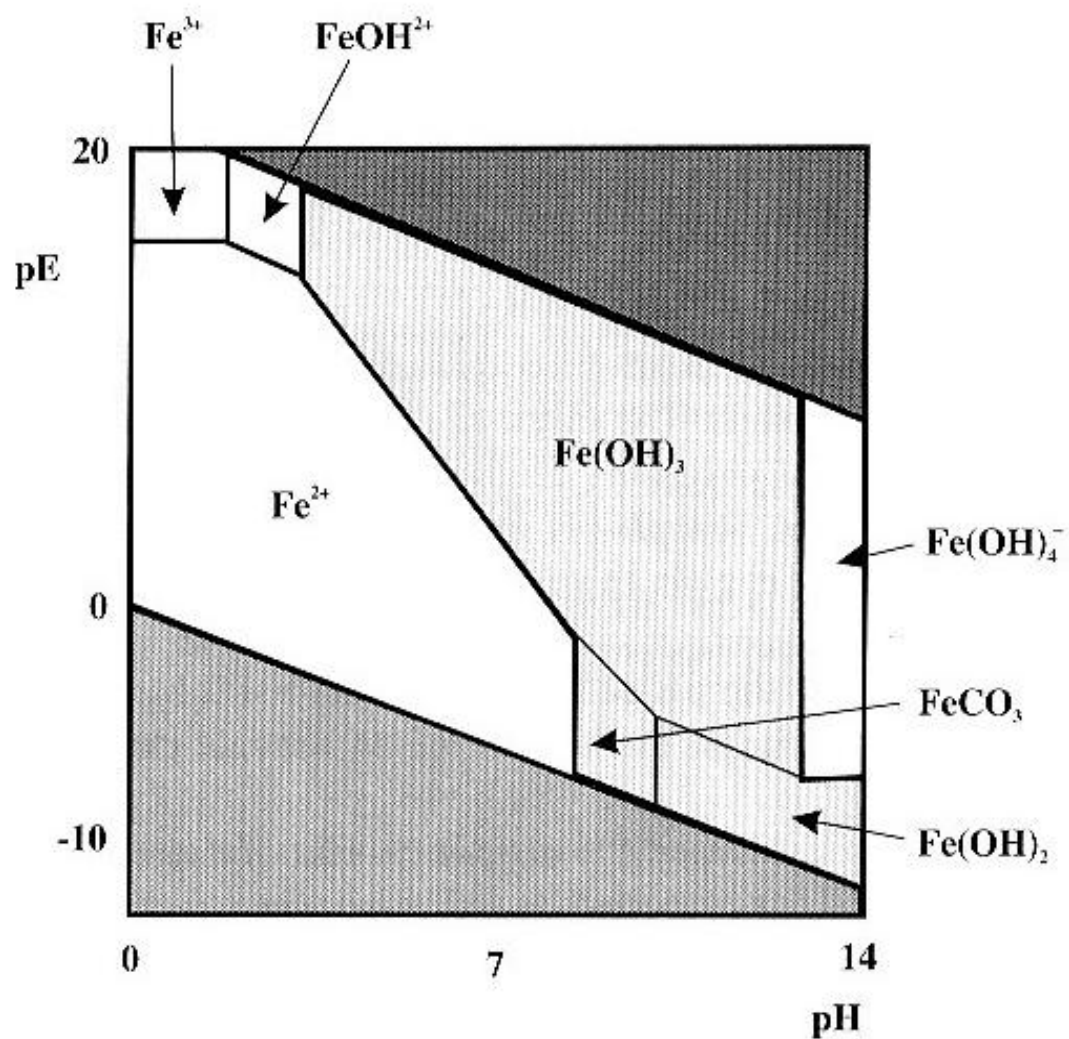


Fig. 1.4. A pE-pH diagram for iron incorporating carbonate species.

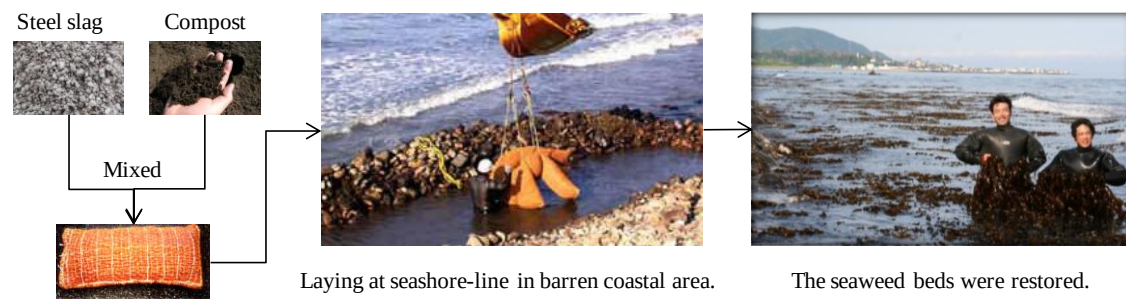


Fig. 1.5. The restoration technique using a fertilizer comprised of steel slag and compost. The mixture of steel slag and compost were packed into bag made by palm fiber. These bags were just embedded at seashore-line in barren coastal area. This approach resulted in the restoration of seaweed beds.

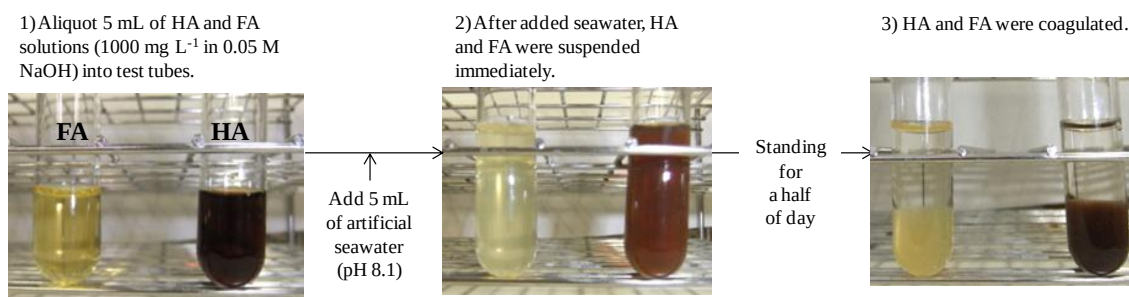


Fig. 1.6. Coagulation of HA (left) and FA (right) in the presence of seawater. Before adding seawater, HA and FA solutions indicated dark brown and yellow colors, respectively. After the coagulation of HA and FA, supernatants turned to clear.

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Chapter 2

***Characterization of seawater extractable
organic matter from bark composts by
TMAH-py-GC/MS***

3.1. Introduction

As mentioned in Chapter 1, barren ground is a phenomenon associated with seaweed depletion in coastal areas, which is a serious problem along the coast of Japan as well as other parts of the world. Our research group focused on the Fe-lacking as the one of reasons for this phenomenon [1], a fertilization technique using steel slag and compost (the detail was shown in Chapter 1) had been attempted and resulted in the restoration of seaweed beds in barren coastal areas [2]. Because the major soluble iron under seawater conditions ($I = 0.7$, pH 8.1) present as the complex species with dissolved organic matter (DOM), in this restoration technique, iron oxides on the surface of the steel slag would be eluted by DOM, which can be extracted from the compost in the fertilizer. Humic (HA) and fulvic (FA) acids are flocculated and/or coagulated in seawater [4-6], although HAs and FAs in the compost have been believed to be able to serve as a chelator of iron in seawater [3]. Figure 1.6 in Chapter 1 just demonstrated that HA and FA in the compost cannot serve as DOM under the presence of seawater. However, DOM fractions in the compost that can contribute to the dissolution of iron from the fertilizer have not been elucidated. Therefore, we focused on seawater extractable organic matter (SWEOM) as a novel DOM fraction from the compost. For the dissolution of Fe from the steel slag in the fertilizer and speciation of Fe in seawater, complexation of Fe with DOM is a key for understanding the mechanical aspects of the technique in the restoration of seaweed beds. The complexing abilities to metal ions of DOM are depending on the structural features. Therefore, the structural features of SWEOM should first be addressed in more detail.

Pyrolysis-gas chromatography/mass spectrometry (py-GC/MS) has been shown to be useful for obtaining detailed structural information on the components of natural organic matter, as well as HAs and FAs [7-11]. This method is based on the thermal breakdown of polymers to variety of subunits that can be separated by gas chromatography and identified by mass spectrometry. In tetramethylammonium hydroxide (TMAH) assisted py-GC/MS (TMAH-py-GC/MS), polar compounds in pyrolysates, such as compounds that contain phenolic hydroxyl groups and carboxylic acids, are transformed in to methyl ester and ether derivatives, which are easily detected by GC/MS. In addition, a variety of side reactions such as cyclization and aromatization reactions of aliphatic chains in HAs during conventional pyrolysis are prevented by adding TMAH [12,13]. Therefore, the TMAH-py-GC/MS has been employed as a useful technique for obtaining detailed structural information related to humic substances from compost samples [14-18]. In this chapter, an SWEOM fraction from a bark compost was extracted with artificial seawater, and its structural features were compared with those for HA and FA fractions extracted from the same compost using TMAH-py-GC/MS in conjunction with variety of spectroscopic methods (UV-vis absorption, FT-IR and solid state CP-MAS ^{13}C NMR spectrometry).

2.2. Materials and Methods

2.2.1. Materials

Tetramethylammonium hydroxide (TMAH), nonadecanoic acid, DOWEXTM HCR-W2 (H^+ -type) cation exchange resin and SperliteTM DAX-8 resin were purchased from Sigma Aldrich (St. Louis, USA). The cation exchange resin was washed 3 times

with 2 M HCl aqueous (solid/liquid = 1/2 ~ 3, v/v) for 24 hours. The washed resin was rinsed with ultrapure water until pH in the supernatant reach to about 7 and then used. The DAX-8 resin was washed by soxhlet extraction with ethanol, acetonitrile and then ethanol for 24 hours. Subsequently, the resin was dried, grained by a mortar and the powder was sieved to 63 ~ 212 μm using the stainless-steel sieves. Prior to use, this DAX-8 resin was rinsed with 0.1 M HCl aqueous and ultrapure water. A compost sample was obtained from the Mori Industry Co., Ltd. (Hokkaido, Japan). This compost was prepared by maturing a mixture of bark tips and cowpats. Prior to use for the test, the compost sample was freeze-dried and filtered through a 2 mm mesh stainless-steel sieve. Compost particles less than 2 mm in size were used in the tests. The elemental composition of the compost was as follows: C 38.8; H 4.86; N 2.45; S 0.43; O 35.5; ash 18.0 (wt %). The artificial seawater was prepared by dissolving the following salts into 1 kg of ultra pure water: NaCl 28.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 6.82 g; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 5.16 g; CaCl_2 1.11 g; KCl 0.725 g; $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ 0.024 g; NaBr 0.084 g; H_3BO_3 0.024 g. Prior to use in the test, the pH of the artificial seawater was adjusted to 8.1 using dilute aqueous NaOH or HCl.

2.2.2. Extraction and purification of HA and FA

The extraction and purification of HA and FA were based on a method approved by the International Humic Substances Society [19,20]. The flowchart for the extraction and purification procedures is shown in Fig. 2.1. The mixture of dry compost and 0.05 M aqueous NaOH (solid/liquid = 1/10, w/w) was shaken under a N_2 atmosphere for 24 hours. Subsequently, the suspension was centrifuged at 10,000 rpm (10397 g) for 15 min, and the supernatant was then filtered through an ADVANTEC 5A filter paper

(collected particle size $> 0.7\ \mu\text{m}$; ash content $< 0.01\%$). Concentrated HCl was added to the suspension to adjust the pH < 1.0 , and the mixture was then stirred for 24 hours. The mixture was centrifuged at $10397\ g$ for 15 min. The slurry of ultrapure water and the precipitated HA (100 mL) was further treated by adding concentrated HCl (3 mL) and HF (2 mL) for 24 hours to remove silicate impurities. After centrifugation ($10397\ g$ for 15 min), the precipitated HA was transferred to a dialysis tube (molecular weight cut-off of 1 kDa) and dialyzed against pure water. Finally, the prepared HA was freeze-dried to give a powder.

The acidified supernatant including FA was passed through a DAX-8 resin column to adsorb the FA. The schematic diagram for the column system of FA purification is shown in Fig. 2.2. The column was rinsed with pure water until the effluent became clear. The adsorbed FA was then eluted with aqueous 0.1 M NaOH. The eluent was then passed through a H^+ -type cation-exchange resin column (DOWEX[®] HCR-W2). Finally, a powdered sample of the H^+ -type FA was obtained by freeze-drying.

2.2.3. Extraction and purification of SWEOM

The flowsheet of the extraction and purification of SWEOM is shown in Fig. 2.3. A mixture of dry compost and artificial seawater at pH 8.1 (solid/liquid = 1/10, w/w) was shaken under a N_2 atmosphere for 3 days. The suspension was then centrifuged at $10397\ g$ for 15 min, and the supernatant was filtered through the ADVANTEC 5A filter paper. The filtrate was then deionized by ultrafiltration with an ultrafiltration membrane filter (molecular weight cut-off of 0.5 kDa, regenerated cellrose, Millipore). The fraction retained was washed with pure water until the effluent water became clear.

Subsequently, the deionized fraction was dialyzed against pure water using a 0.5 kDa dialysis tube. After the dialysis, the slurry in the dialysis tube was freeze-dried, and a powdered sample of SWEOM was obtained. The yields of samples were calculated as:

$$\text{Yield (g kg}^{-1}\text{)} = \{\text{Sample obtained finally (g)}\} / \{\text{Compost taken initially (kg)}\}.$$

2.2.4. Analyses of SWEOM, HA and FA

2.2.4.1. Analyses of elemental composition and amino acid contents

Elemental compositions (C, H, N, S and ash) and amino acid content were determined at the Center for Instrumental Analysis at Hokkaido University [21]. The percent of oxygen was calculated by subtracting the sum of the percent C, H, N, S and ash from 100%. A 10 mg portion of a powdered sample was hydrolyzed in 6 M HCl at 110 °C for 24 h, and the resulting amino acids were separated on an ion-exchange column and then detected by the ninhydrin reaction [21].

2.2.4.2. Measurement of acidic functional group contents

The total acidity and carboxylic acid content were determined by a titration method, which were carried out using an AUT-501 type auto titration system (TOA DKK) connected to GST-5311 C type glass electrode (TOA DKK). The ultrapure water was boiled for 1 ~ 2 hours and cooled under N₂ bubbling to remove dissolved CO₂. The water was preserved by seal with soda lime tube, and used as CO₂-free water.

Total acidity was determined by the Ba(OH)₂ method [22]. 10 mg portion of powdered samples were weight to 50 mL Erlenmeyer flasks, and 20 mL of 0.1 M Ba(OH)₂ aqueous was then added. To avoid the contamination of CO₂ from air, flasks were sealed with soda lime tube. Subsequently, the flasks were allowed to shake under

darkness at room temperature for 24 hours. After shaking, sample solutions were filtered through a glass membrane filter (0.45 μm) prewashed with CO_2 -free water. The flask and residue on the filter were washed 3 times with CO_2 -free water. Subsequently, the filtrate was immediately titrated with 0.1 M HCl aqueous until the pH reaches to 8.4 under N_2 atmosphere. The titrant (0.1 M HCl) was standardized using 0.0125 M of Na_2CO_3 aqueous as a primary standard. The total acidity was calculated as:

$$\text{Total acidity (mmol g}^{-1} \text{ of C)} = \frac{(V_{\text{blank}} - V_{\text{sample}}) \times [\text{HCl}]}{\text{sample (mg)} \times \%C/100} \times 1000$$

where V_{blank} and V_{sample} represent the titrated volume (mL for 0.1 M HCl aqueous) for blank (i.e., 0.025 M $\text{Ba}(\text{OH})_2$ aqueous alone) and sample, respectively. The carboxyl group content was determined by $\text{Ca}(\text{CH}_3\text{COO})_2$ method [22]. 10 mg portion of the powdered sample was weighted to a 100 mL Erlenmeyer flask, and 10 mL of 0.2 M $(\text{CH}_3\text{COO})_2\text{Ca}$ solution and 40 mL of CO_2 -free water added. After shaking for 24 hours and filtering through the 0.45 μm filter, the filtrate was titrated with 0.05 M NaOH aqueous up to pH 9.8 under N_2 atmosphere. The 0.05 M NaOH aqueous as the titrant was standardized with the standardized 0.1 M HCl aqueous as a secondary standard. The carboxylic acid content was calculated as:

$$\text{COOH content (mmol g}^{-1} \text{ of C)} = \frac{(V_{\text{sample}} - V_{\text{blank}}) \times [\text{NaOH}]}{\text{Sample (mg)} \times \%C/100} \times 1000$$

where V_{blank} and V_{sample} represent the titrated volume (mL for 0.05 M NaOH aqueous) for blank (i.e. 0.2 M $(\text{CH}_3\text{COO})_2\text{Ca}$ aqueous alone) and samples, respectively. The analyses were conducted to duplicate. The phenolic hydroxyl group content was calculated by subtracting the carboxyl group content from the total acidity.

2.2.4.3. Measurement of molecular weight

The number-average (M_n) and weight-average (M_w) molecular weights of the materials were estimated by size exclusion chromatography, as described in a previous report [23]. 2,600, 990, 350, 150, 77, 49, 32, 17, 13, 6.6, 4.3, 1.4 and 0.208 kDa of polystyrenesulfonate sodium salts (PSSA, Sigma Aldorich) were used as standard materials. Void volume (ca. 6.7 mL) was determined by the retention time for 2,600 kDa of the standard. The calibration of molecular size was based on the non-linear least square regression analysis of the data set for molecular weight of PSSA and retention time to Boltzmann equation, as described below:

$$\log M = \frac{A_1 - A_2}{1 + e^{(x - x_0)/dx}} + A_2$$

where M and x represent molecular weight of PSSA and retention time, respectively. A_1 and A_2 represent constant values, which can be calculated by the curve-fitting. A 20 μ L aliquot of a 250 mg L⁻¹ sample solution, which was diluted with phosphate buffer (pH 7), was injected into Jasco PU-2080 Plus type HPLC system (Japan Spectroscopic Co., Ltd). The mobile phase consisted of a mixture of 0.01 M phosphate buffer (pH 7.0) and acetonitrile (75/25 = v/v), and the flow rate was set at 0.75 mL min⁻¹. The column temperature was maintained at 40 °C, and a UV-2075 UV-vis detector (Japan Spectroscopic Co., Ltd.) was used for detection of standard and sample at 260 nm of detection wavelength. A TSKgel Alpha-M rigid porous polymer gel column (7.8 mm i.d. $\times$ 300 mm, TOSOH Co., Ltd) was used as the solid phase. The analysis was conducted to triplicate. The number average (M_n) and weight average (M_w) molecular weights were calculated as follows:

$$M_n = \Sigma h_i / \{ \Sigma (h_i / M_i) \},$$

$$M_w = \Sigma (h_i \times M_i) / \Sigma h_i,$$

where M_i and h_i are the molecular weight as referred to the calibration of standard

samples and the peak height at i -th retention volume, and the value of the M_w/M_n ratio represents polydispersity in samples. The M_n and M_w are summarized in Table. 2.2.

2.2.4.4. UV-vis, FT-IR and solid state CP-MAS ^{13}C NMR spectrometry

Spectra of FT-IR and UV-vis absorption for HA, FA and SWEOM were recorded as described in a previous report [23]. FTIR spectra were collected using an FT/IR 4100-type spectrometer (Japan Spectroscopic Co. Ltd.) with KBr pellets (sample/KBr = 1/99, w/w). Spectra were recorded between 4000 and 400 cm^{-1} at intervals of 1 cm^{-1} . The wavenumber dissolution was set at 4 cm^{-1} . UV-vis absorption spectra were collected using a V-630 type UV-vis spectrometer (Japan Spectroscopic CO., Ltd), and absorbance values were converted to absorptivity using the equation below:

$$\text{Absorptivity} (\text{L g}^{-1} - \text{C cm}^{-1}) = \frac{\text{Absorbance}}{[\text{DOM}(\text{g L}^{-1})] \times \frac{\% \text{C}}{100} \times d (\text{cm})}$$

where [DOM] and d represent the concentration of HA, FA and SWEOM, and light pass length (1 cm). CP-MAS solid state ^{13}C NMR spectra were collected using a CMX-300 type NMR spectrometer (75.6 MHz, Chemagnetics) under following conditions: spinning rate 10 kHz, contact time 1 ms, pulse delay 4 s, integration times 16000.

2.2.4.5. Analysis of TMAH-py-GC/MS

A 1.0 ± 0.1 mg portion of the powdered sample was placed in a 50 μL deactivated stainless-steel cup. A 25 μL aliquot of TMAH in methanol (40 mg mL^{-1}) and a 10 μL aliquot of nonadecanoic acid in acetone (0.06 mg mL^{-1}) as an internal standard (ISTD) were then added to the cup. After removing the solvents under reduced pressure, the cup was introduced into a PY-2020D type Double-Shot Pyrolyzer (Frontier Laboratories

Ltd.) connected to a Shimadzu GC-17A/QP5050 type GC/MS system. Helium (99.995 % purity) was used as the carrier gas, and flash pyrolysis of the powdered samples were carried out at 550 °C for 0.4 min. A Quadrex 100 % dimethylpolysiloxane capillary column (0.25 mm i.d. × 25 m, 0.25 µm film thickness) was employed in the separation of the pyrolysate compounds. The temperature program for the GC oven was as follows: 50 °C for 1 min; 50 – 300 °C at a heating rate of 5 °C min⁻¹; 300 °C for 4 min. To compare the peak areas for each sample, the relative peak areas (%) were calculated on the basis of the ratio of the peak area for each pyrolysate compound to that for ISTD.

2.3. Results and Discussion

2.3.1. Characterization by TMAH-py-GC/MS

2.3.1.1. Assignment of pyrolysate compounds

Pyrograms for the TMAH py-GC/MS are shown in Fig. 2.4. The pyrolysate compounds were identified by mass spectrometry, and assigned to various groups as follows: (A) furan derivatives; (B) nitrogen-containing compounds; (C) methoxy- and hydroxy-benzenes; (D) aromatic ketones and aldehydes; (E) phenolic acids and benzoic acids derivatives; (F1) saturated fatty acids; (F2) unsaturated fatty acids; (G) sterols; (H) terpenoids; (I) others. The sums of the relative peak areas for each group are shown in Fig. 2.5. In addition, the pyrolysate compounds identified from HA, FA and SWEOM are summarized in Table 2.1.

As shown in Fig. 2.4, numerous pyrolysate compounds were detected in the SWEOM and HA. Aromatic compounds were detected in HA as major pyrolysate

compounds. In the SWEOM, fatty acids were the predominant pyrolysate compounds and the sterol content was much larger than those for HA and FA.

2.3.1.2. Heterocyclic carbonyl and nitrogen-containing compounds

Furan derivatives (A) such as furans and/or furanones, which are pyrolysate compounds that are produced from polysaccharides [24,25], were found only in FA. Furans are known to be pyrolysate compounds derived from polysaccharides, and difficult to detect using TMAH pyrolysis [24-26]. However, large amounts of furans derived from cyclodextrins were detected as pyrolysate compounds by TMAH-py-GC/MS [27]. To check whether furans were further detected in the samples or not, py-GC/MS analysis without TMAH was carried out. Using TMAH-py-GC/MS (Fig. 2.4 and Table 2.1), 2,4-furandicarboxylic acid was detected as a pyrolysate compound of FA. However, when py-GC/MS was used without TMAH (see Fig. 2.5), 2-furancarboxaldehyde and 5-methyl-2-furancarboxyaldehyde were detected in SWEOM, as well as FA. Thus, SWEOM appears to contain some saccharide moieties, but the amounts appear to be relatively lower, compared to whole pyrolysate compounds that were detected by TMAH-py-GC/MS. Polysaccharides in plant tissue (e.g., cellulose) are known to be transformed into olefins and/or aromatic moieties via oxidation during composting [28]. It is known that mature compost contains larger amounts of HA with a higher aromaticity [23]. Thus, the HA was a more aromatic character than FA and SWEOM, primarily due to the biodegradation of saccharides in the starting materials.

The nitrogen-containing pyrolysate compounds (B) are produced from amino acids, peptides, proteins and nucleic acids in HA, FA and SWEOM [14,29]. As shown in

Table 2.1, large amounts of indole and pyrrolidine were detected in all samples. The major pyrolysate compounds in group B were methylindole (peaks 12 and 17 in Table 2.1) (22 and 60 % in B for SWEOM and HA, respectively) and dimethylindole (peak 15 in Table 2.1) (11 % in B for SWEOM). The pyrolysate compounds containing indole structures are derived from tryptophan. Pyrrolidine pyrolysate compounds (peaks 4 and 18 in Table 2.1) can be derived from proline or from porphyrins in terrestrial plants [30]. Large amounts of uracil, which are of RNA origin [31], were detected in FA (peaks 16 and 19).

2.3.1.3. Aromatic compounds

Pyrolysate compounds C – E included aromatic moieties, which are mainly derived from lignin [14,32-35]. Phenolic acid pyrolysate compounds (E), such as methoxybenzoic acids (peaks 20, 23 and 24) and methylphenol (peak 7), were found in both HA and SWEOM. These compounds were derived from wood lignin [11,14,15,32-35]. It has, however, been reported that some types of pyrolysate compounds such as for benzenecarboxylic acids cannot be originally present in the structure of the natural organic matter, such as HA and SWEOM, but rather are formed via a Cannizzaro reaction from benzaldehyde derivatives [36,37]. Thus, the contents of benzenecarboxylic acid in the pyrolysates are not consistent with carboxylic contents listed in Table 2. Vanillic acid (peak 20), which is detected in all samples, is known to be derived from wood lignins from conifer species or aromatic domains of suberin in plant cell walls [32]. As shown in Table 1, methylphenols (peak 7 and 9) and trimethoxybenzoic acid methyl ester (peak 24) were distinct feature of HA. Although the levels of aromatic pyrolysate compounds in SWEOM were smaller than those of HA,

methylphenol, di- and tri-methoxy benzoic acid methyl esters were dominant, similar to HA. Aromatic ketones and aldehydes (D) were little detected in SWEOM. These results suggest that lignin fractions, major component in HA [38], are not readily soluble in seawater.

2.3.1.4. Lipids

Lipid pyrolysate compounds (F1, F2, G and H) are known to be structural units of humic substances from microbial origins and waxes in wood bark [14,32,38,39]. Fatty acids in natural organic matter are not always present as free acids. Fatty acid pyrolysate compounds detected by TMAH-py-GC/MS can be produced from the cleavage of ester-linkages as a result of pyrolysis [12,39-41]. The distributions of F1 and F2 type pyrolysate compounds for HA, FA and SWEOM are shown in Fig. 2.7. In general, fatty acids are hydrophobic molecules and are not readily soluble in water. Nevertheless, the levels of saturated and unsaturated fatty acids in SWEOM were much higher than those for HA and FA. It is particularly noteworthy that the levels of hexadecanoic and octadecanoic acids, which are components of lecithin in phospholipids [29,40,42], were much higher in SWEOM, compared to HA and FA (Fig. 2.7). In the FA, however, higher levels of octadecenoic acid and lower levels of hexadecanoic acid were detected. The former can also be produced from suberin [43]. These findings suggest that the octadecenoic acid pyrolysate compound in FA was not derived from phospholipids, but from suberin a component of the plant cuticle [43]. The levels of sterols pyrolysate compounds for SWEOM were much higher than those for HA and FA. These compounds are found in higher plants [14,40,43] and are considered to be derived from conifer bark in a compost.

2.3.2. Elemental compositions, amino acid contents and molecular weights

Elemental compositions and molecular weights of the HA, FA and SWEOM are summarized in Table 2.2. The H/C and N/C atomic ratios for SWEOM were significantly larger than those for HA. These results suggest that SWEOM contains larger amounts of saturated hydrocarbons and nitrogen-containing compounds, such as peptides and proteins [3,14,32]. The O/C atomic ratio for SWEOM was comparable to that of FA. This suggests that the SWEOM contains higher levels of acidic functional groups, such as FA. Alternatively, large amounts of oxygen-containing functional groups, such as ethers and esters, may be included in SWEOM. The M_w value and the M_w/M_n ratio for SWEOM were significantly larger than those for HA and FA. These results suggest that the SWEOM is comprised of a mixture of macromolecules with a range of molecular size. The fractionation of SWEOM from seawater matrices was based on ultrafiltration. Thus, SWEOM includes non-humic materials, such as higher molecular weight proteins as well as humic fractions. To estimate the amount of protein-like components in HA, FA and SWEOM, the amino acids obtained by hydrolysis of the three samples were analyzed. As shown in Table 2.3, total amino acid content for the SWEOM (15.3 mg g^{-1}) was much larger the corresponding values for HA (6.23 mg g^{-1}) and FA (1.69 mg g^{-1}). While the compositions of amino acids for SWEOM were not so different from that for HA, the glutamic acid and glycine compositions in FA (15.0 and 30.8 %, respectively) were much higher than those for SWEOM (10.5 and 11.9 %, respectively) and HA (12.1 and 13.0 %, respectively).

2.3.3. UV-vis absorption spectra

The UV-vis absorption spectra of HA, FA and SWEOM are shown in Fig. 2.8. The absorptivity for the HA was higher than those for FA and SWEOM at all wavelengths. It is known that absorption in the UV region (200-400 nm) can be attributed to the electron transitions for $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ of carbonyl C=O bonds and C=C bonds [44,45]. The absorptivity at 280 nm is a well known index of the aromaticity for humic substances [46-48]. The absorptivities in the UV region (200-360 nm) for HA and FA were higher than that for SWEOM, indicating that HA and FA contain higher aromatic and carbonyl moieties than the SWEOM. These results suggest that SWEOM has less aromatic characteristics than HA and FA.

2.3.4. FT-IR spectra

Figure 5 shows FT-IR spectra for HA, FA and SWEOM. The spectral bands were assigned as follows [45,48-51]: broad spectral band at 3400 – 3000 cm^{-1} for OH stretching; peaks at 2850 – 2950 cm^{-1} for asymmetric and symmetrical stretching of methyl and ethylene C-H bonding; peak or shoulder at 1720 cm^{-1} for C=O stretching of COOH and/or ketones; peak or shoulder at 1650 cm^{-1} for C=O stretching of amide-I band or aromatic C=C stretching; the band at 1550 cm^{-1} for N-H bending of amide-II band; spectral bands at 1220 – 1230 cm^{-1} for C-O stretching and OH bending of carboxylic acids, alcohols, esters and ethers; the peak at 1085 – 1150 cm^{-1} assigned to C-O-C stretching of ethers or C-O stretching for aliphatic alcohols.

The intensity of the peak at 1720 cm^{-1} for FA was stronger than those for SWEOM and HA, suggesting that FA contains higher contents of carboxylic acids. The peaks at 2850 – 2950 cm^{-1} for the SWEOM were more intense than those for HA and FA. This

indicates that SWEOM has more aliphatic characteristics than HA and FA, which is in agreement with the H/C atomic ratios and UV-vis absorption spectra. In addition, the strong band at about 1050-1100 cm^{-1} for SWEOM suggests that higher levels of ethers and aliphatic alcohols are present. These results are supported by the larger O/C ratio and the smaller acidic functional group contents in SWEOM (Table 2.2). The bands at 1650 and 1550 cm^{-1} in the spectrum of the SWEOM was assigned as an amide-I and -II band, respectively [51]. This suggests that the SWEOM structure is enriched in amino acids and/or proteins, again, being consistent with the total amino acid content (Table 2.3).

2.3.5. Solid state CP-MAS ^{13}C NMR spectra

The NMR spectra and the percents of carbon species for the SWEOM, HA and FA, which were calculated from the peak integration values in the solid-state CP-MAS ^{13}C NMR spectra, are shown in Fig 2.10. The peaks were assigned as follows [54]: alkyl carbons (0 – 60 ppm); aliphatic alcohols and ethers carbons (60 – 90 ppm); anomeric carbons (90 – 105 ppm); aromatic carbons attached to proton or carbon (105 – 135 ppm); aromatic carbons attached to oxygen (135 – 160 ppm); carbonyl carbons (acids, esters and amides) (160 – 180 ppm); carbonyl carbons (quinones, ketones and aldehydes) (180 – 220 ppm). The peaks for aliphatic alcohols and ethers at 60 – 90 ppm for SWEOM were much more intense than those for HA and FA. These results are consistent with the larger H/C ratios for these materials (Table 2.2) and the enhanced peaks for alcohol and ether C-O stretching in FT-IR spectra of the SWEOM (Fig. 2.9). For the spectrum of HA, the enhanced peak appeared at 135 – 160 ppm, which is assigned to aromatic carbon attached with oxygen, such as lignin units. For SWEOM,

the carbon species at this peak was much smaller than that for HA (8.25% in SWEOM, 15.1% in HA), being consistent with the result the level of phenolic-OH for SWEOM was smaller than that of HA (Table 2.2). In addition, the peak integration value for aromatic carbon (105 – 160 ppm) for the SWEOM was also smaller than those for HA and FA, supporting the conclusion that the SWEOM has more aliphatic characters.

2.3.6. Structural features of SWEOM

The nitrogen content, N/C molar ratio and total amino acid contents for SWEOM were significantly larger than those for HA and FA (Table. 2.3). However, the levels of nitrogen-containing pyrolysate compounds (B) were not correlated with the nitrogen contents, N/C ratios and total amino acid contents in HA, FA and SWEOM. In TMAH-py-GC/MS, indole pyrolysate compounds (peaks 12, 14 and 17 in Fig. 2.4) are formed by the pyrolysis of tryptophan, and cannot be considered as quantitatively representative of the amino acid composition of a material [21,29]. The numerous of amide bands in FT-IR spectra of SWEOM (Fig. 2.9) are consistent with the trends in nitrogen contents, N/C ratios and total amino acid contents (Table 2.3). These results suggested that nitrogen-containing compounds in SWEOM are mainly present as amide forms relating to proteins and peptides.

In addition, as shown in Table 2.2, the carboxylic and phenolic hydroxyl group contents for SWEOM were significantly lower than those for HA and FA. Thus, the higher contents of fatty acid pyrolysate compounds in SWEOM suggest that fatty acids in SWEOM are largely present in the form of esters. Thus, the detected acids and alcohols (and/or phenols) may be due to the base-catalyzed hydrolysis of ester-linkages in TMAH. These results suggest that the extent of Fe-binding for SWEOM is less than

that for HA and FA, being consistent with the trends in Fe(II) binding capacities of these fractions [52]. Furthermore, phosphoric acid trimethyl ester (peak 2 in Fig. 2.4), derived from the cleavage of phospholipids, was detected only the case of SWEOM (Table 2.1). From this result, indicated that phospholipids probably are soluble in seawater, and a portion of them are involved in SWEOM. The larger M_w of SWEOM may be then due to dissolved phospholipid that becomes aggregated during the extraction process under the high ionic strength conditions used [53]. In addition, some of the ester-linkages may have been hydrolyzed during the isolation of the HAs, which involve alkaline extraction and acid treatments [21]. Thus, it is not possible to discriminate whether the detected fatty acids for HA and FA are derived from esters or free acid.

2.4. Conclusions

For the slag/compost fertilizer to restore seaweed-bed in barren coastal areas, HA and FA cannot be used because they become flocculated in seawater. Thus, the focus of this study was on SWEOM as a novel fraction of DOM from compost, and the structural features of the SWEOM were compared with HA and FA derived from the same origin. Elemental analysis, amino acid analysis, acidic functional group analysis and spectroscopic measurements show that SWEOM has more aliphatic characteristics, which contained the higher level of proteins and the less carboxylic acids than HA and FA. The TMAH-py-GC/MS analyses suggest that the higher levels of fatty acid pyrolysate compounds in SWEOM can be attributed to the cleavage of ester-linkages of phospholipids as a result of pyrolysis.

Table 2.1. List of the assigned pyrolysate compounds and their relative peak areas for HA, FA and SWEOM.

Peak No.	Compounds	Group ^b	Relative peak areas ^c		
			SWEOM	HA	FA
1	1-Methoxy-4-methylbenzene	C	n.d. ^a	26.6	n.d. ^a
2	Phosphoric acid trimethyl ester	I	48.7	n.d. ^a	n.d. ^a
3	Butanedioic acid dimethyl ester	F1	n.d. ^a	n.d. ^a	189
4	1-Methyl-2,5-pyrrolidinedione	B	n.d. ^a	17.3	n.d. ^a
5	4-Methoxyphenol	C	n.d. ^a	56.9	n.d. ^a
6	1-Methoxy-4-methylbenzene	C	75.17	n.d. ^a	n.d. ^a
7	4-Methylphenol	C	24.9	76.1	n.d. ^a
8	Glutaric acid dimethyl ester	F1	n.d. ^a	n.d. ^a	22.7
9	2-Methoxy-4-methylphenol	C	n.d. ^a	58.1	n.d. ^a
10	4-Methoxycarbonyl-4-butanolide	A	n.d. ^a	n.d. ^a	22.6
11	Hexanedioic acid dimethyl ester	F1	n.d. ^a	n.d. ^a	43.1
12	1-Methylindole	B	38	55.2	n.d. ^a
13	2-Methoxy-1,3-benzenediol	C	n.d. ^a	82.8	n.d. ^a
14	2,4-Furandicarboxylic acid dimethyl ester	A	n.d. ^a	n.d. ^a	9.39
15	1,3-Dimethylindole	B	26.1	n.d. ^a	n.d. ^a
16	1,3-Dimethyluracil	B	n.d. ^a	n.d. ^a	70.5
17	3-Methyl-1H-indole	B	12.7	61.3	n.d. ^a
18	N-(4-methyl-3-pentenyl)-pyrrolidine	B	n.d. ^a	n.d. ^a	60.5
19	1,3,5-Trimethyluracil	B	n.d. ^a	n.d. ^a	51.4
20	Vanillic acid methyl ester	E	21	53.2	13.4
21	Dodecanoic acid methyl ester	F1	25.7	13.7	9.76
22	3,5-Dimethoxyacetophenone	D	n.d. ^a	27.5	n.d. ^a
23	3,4-Dimethoxybenzoic acid methyl ester	E	31.5	78.5	n.d. ^a
24	3,4,5-Trimethoxybenzoic acid methyl ester	E	27	45.5	n.d. ^a
25	3,4,5-Trimethoxybenzyl methyl ether	C	33.9	38.4	n.d. ^a
26	9-Methyltetradecanoic acid methyl ester	F1	116	12.1	n.d. ^a
27	12-Methyltetradecanoic acid methyl ester	F1	82.5	n.d. ^a	n.d. ^a
28	Hexadecenoic acid methyl ester	F2	82	n.d. ^a	n.d. ^a
29	Hexadecanoic acid methyl ester	F1	161	26.4	5.48
30	Heptadecanoic acid methyl ester	F1	50.7	n.d. ^a	n.d. ^a
31	14-Methylhexadecanoic acid methyl ester	F1	51.8	n.d. ^a	n.d. ^a
32	2-Hexylcyclopropaneoctanoic acid methyl ester	F1	48.5	n.d. ^a	n.d. ^a
33	Octadecenoic acid methyl ester	F2	70.1	11.9	45.2
34	Octadecenoic acid methyl ester	F2	84.5	n.d. ^a	172
35	Octadecanoic acid methyl ester	F1	104	18.8	34.39
36	Isopimaric acid methyl ester	H	12.3	9.63	n.d. ^a
37	Docosanoic acid methyl ester	F1	42.4	n.d. ^a	n.d. ^a
38	Tetracosanoic acid methyl ester	F1	30.2	10.1	n.d. ^a
39	Stigmasteran-3,5-diene	G	36.2	11.6	5.89
40	β -Sitosterol	G	36.2	9.72	n.d. ^a

^a Not detected.^b (A) furan derivatives; (B) nitrogen-containing compounds; (C) methoxy- and hydroxy-benzenes; (D) aromatic ketones and aldehydes; (E) phenolic and benzoic acid derivatives; (F1) saturated fatty acids; (F2) unsaturated fatty acids; (G) sterols; (H) terpenoids; (I) others.^c Values for each compound are 100-fold of relative areas to ISTD.

Table 2.2. Elemental compositions, molecular weights and acidic functional group contents for HA, FA and SWEOM.

	SWEOM	HA	FA
Yields (g kg ⁻¹)	1.20	58.0	1.90
Elemental compositions (w %)			
C	45.7 ± 0.5	51.9 ± 0.9	45.3 ± 0.4
H	6.27 ± 0.16	5.26 ± 0.64	4.50 ± 0.45
N	6.41 ± 0.12	5.11 ± 1.75	3.85 ± 0.27
O	38.1 ± 0.3	28.8 ± 3.2	41.6 ± 2.0
S	0.84 ± 0.04	8.54 ± 4.28	1.69 ± 1.71
Ash	2.74 ± 0.20	0.41 ± 0.28	3.06 ± 1.71
Atomic ratio			
H/C	1.64 ± 0.06	1.21 ± 0.16	1.18 ± 0.11
N/C	0.12 ± 0.00	0.08 ± 0.03	0.07 ± 0.01
O/C	0.63 ± 0.01	0.42 ± 0.04	0.69 ± 0.04
Molecular weights			
M_w^a	28700 ± 500	10900 ± 200	1250 ± 20
M_w/M_n^b	17.7	7.02	1.62
Acidic functional group contents (mmol g ⁻¹ C)			
-COOH	3.85 ± 0.01	4.80 ± 0.12	15.5 ± 0.3
Phenolic-OH	3.75 ± 0.86	5.90 ± 1.52	3.64 ± 1.10

^a weight-average molecular weight.^b number-average molecular weight.

Table 2.3. Percentages of amino acid residues as a result of the hydrolysis of HA, FA and SWEOM.

Amino acids	Content of amino acid (mg g ⁻¹ -Sample)		
	SWEOM	HA	FA
Alanine (Ala)	1.88	0.696	0.226
Arginine (Arg)	0.512	0.200	n.d. ^a
Asparagine (Asn)	n.d. ^a	n.d. ^a	n.d. ^a
Aspartic acid (Asp)	1.77	0.709	0.269
Cysteine (Cys)	0.095	0.042	0.026
Glutamine (Gln)	n.d. ^a	n.d. ^a	n.d. ^a
Glutamic acid (Glu)	1.60	0.755	0.254
Glycine (Gly)	1.82	0.807	0.521
Histidine (His)	0.202	0.075	0.003
Isoleucine (Ile)	0.790	0.329	0.041
Leucine (Leu)	1.28	0.519	0.057
Lysine (Lys)	0.460	0.202	0.010
Methionine (Met)	0.165	0.049	0.018
Phenylalanine (Phe)	0.622	0.243	0.016
Proline (Pro)	0.730	0.354	0.050
Serine (Ser)	0.805	0.304	0.037
Threonine (Thr)	1.02	0.357	0.059
Tryptophan (Trp)	n.d. ^a	n.d. ^a	n.d. ^a
Tyrosine (Tyr)	0.388	0.116	0.013
Valine (Val)	1.14	0.472	0.090
Total amino acids content	15.3	6.23	1.69

^a Not detected.

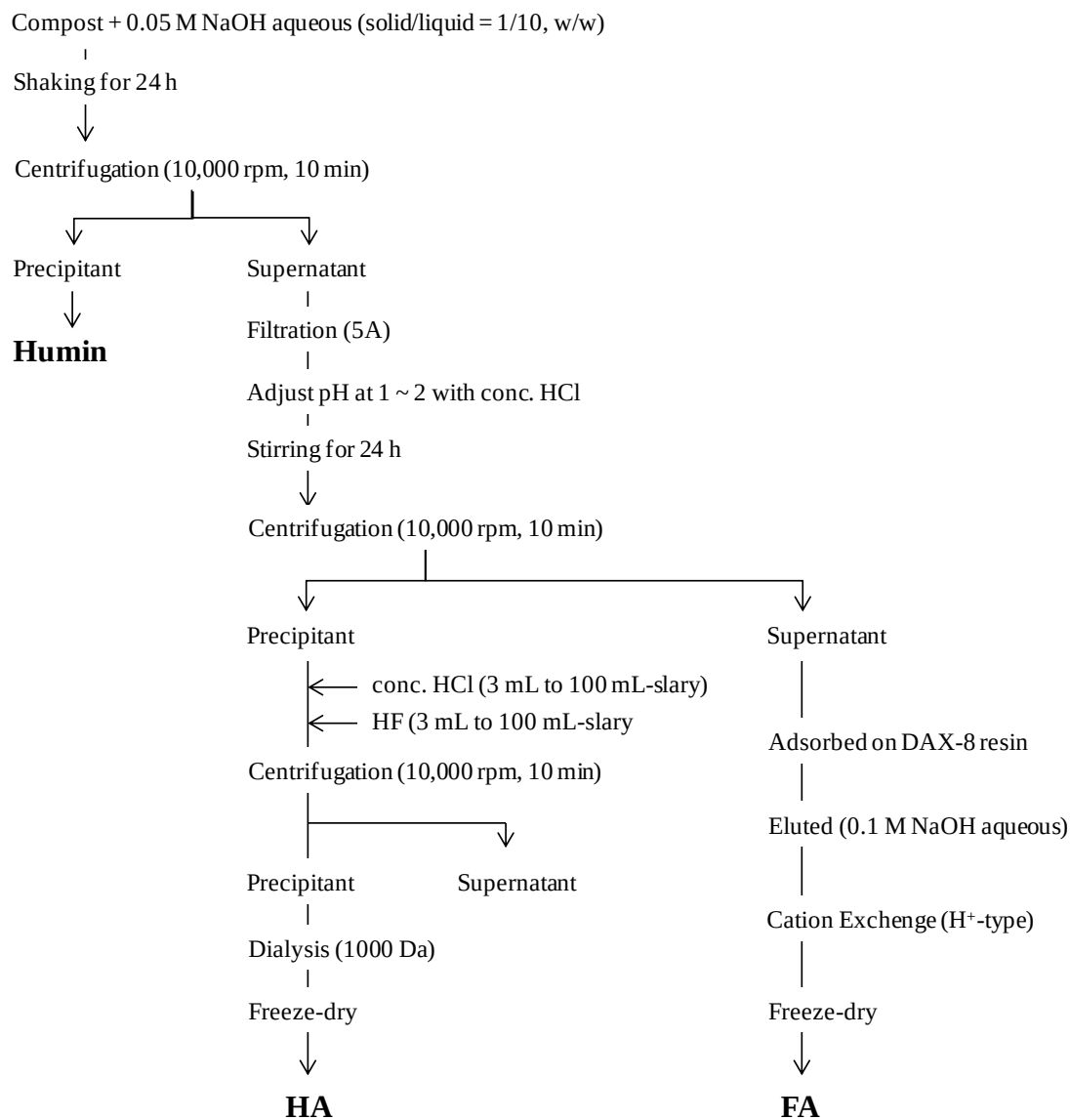


Fig. 2.1. Flowsheet of extraction method of HA and FA.



Fig. 2.2. Schematic diagram for the column system. A: DAX-8 resin column, B: Cation exchange column, C: Pump to draw solution into column. This picture shows the state of adsorbing DOM on DAX-8 resin. The DOM solution has yellow color (lower right), and the drain solution is clear (lower left).

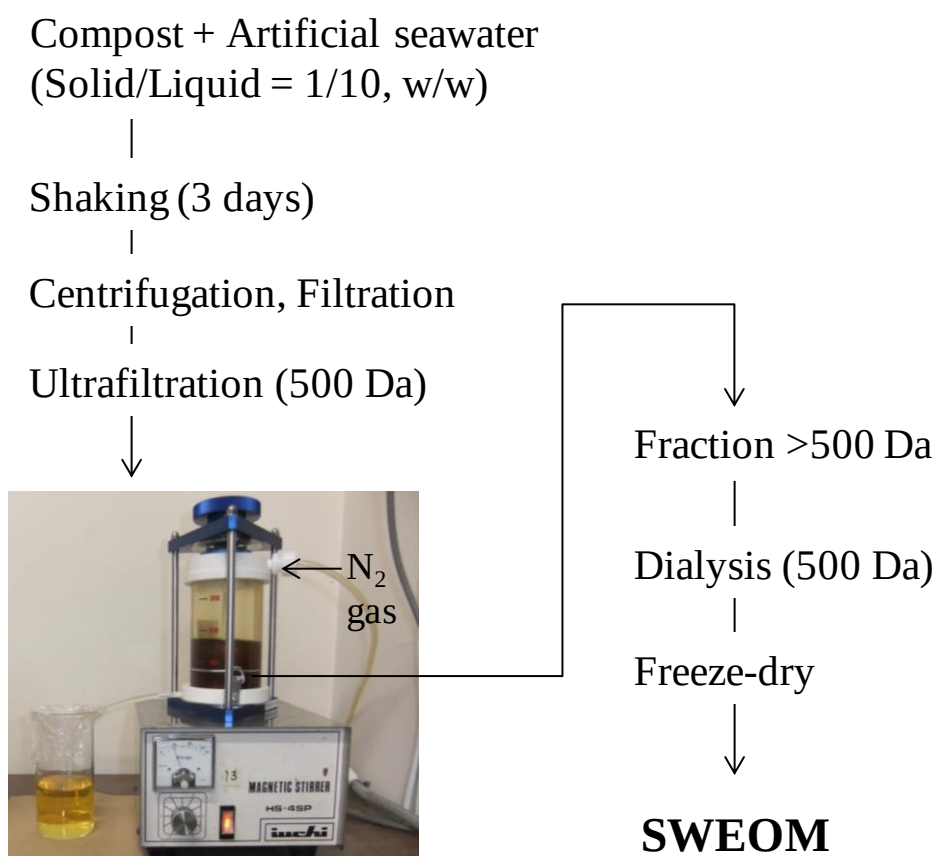


Fig. 2.3. Flowsheet of extraction method of SWEOM

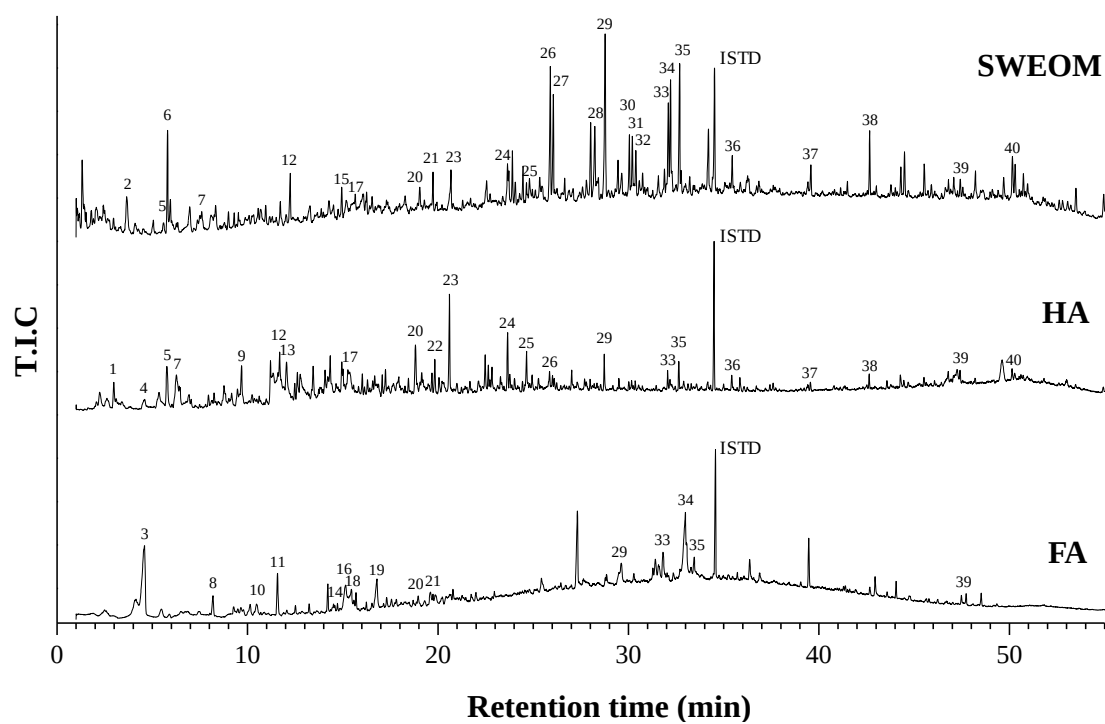


Fig. 2.4. Pyrograms by TMAH-py-GC/MS for HA, FA and SWEOM.

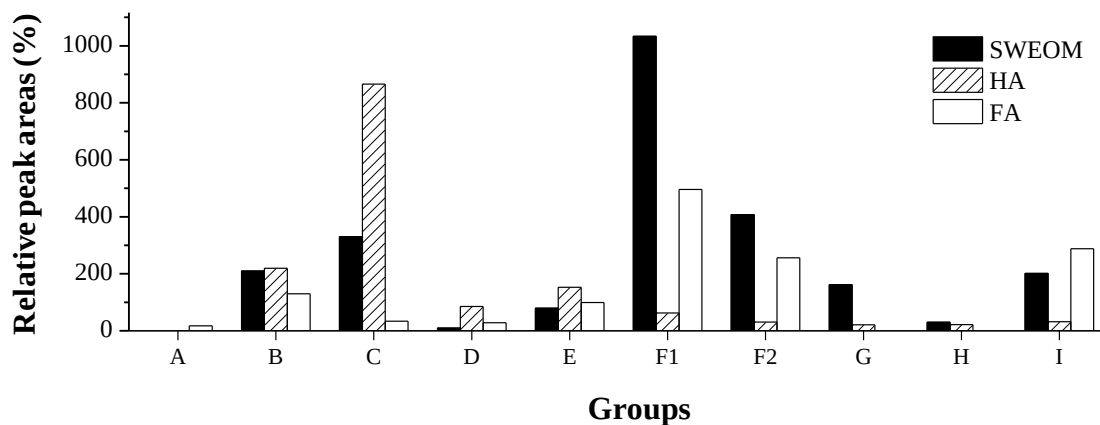


Fig. 2.5. The total relative peak areas of classified pyrolysate compounds: (A) furan derivatives; (B) nitrogen-containing compounds; (C) methoxy- and hydroxy-benzenes; (D) aromatic ketones and aldehydes; (E) phenolic and benzoic acid derivatives; (F1) saturated fatty acids; (F2) unsaturated fatty acids; (G) sterols; (H) terpenoids; (I) others. Each value is percentage of ISTD areas.

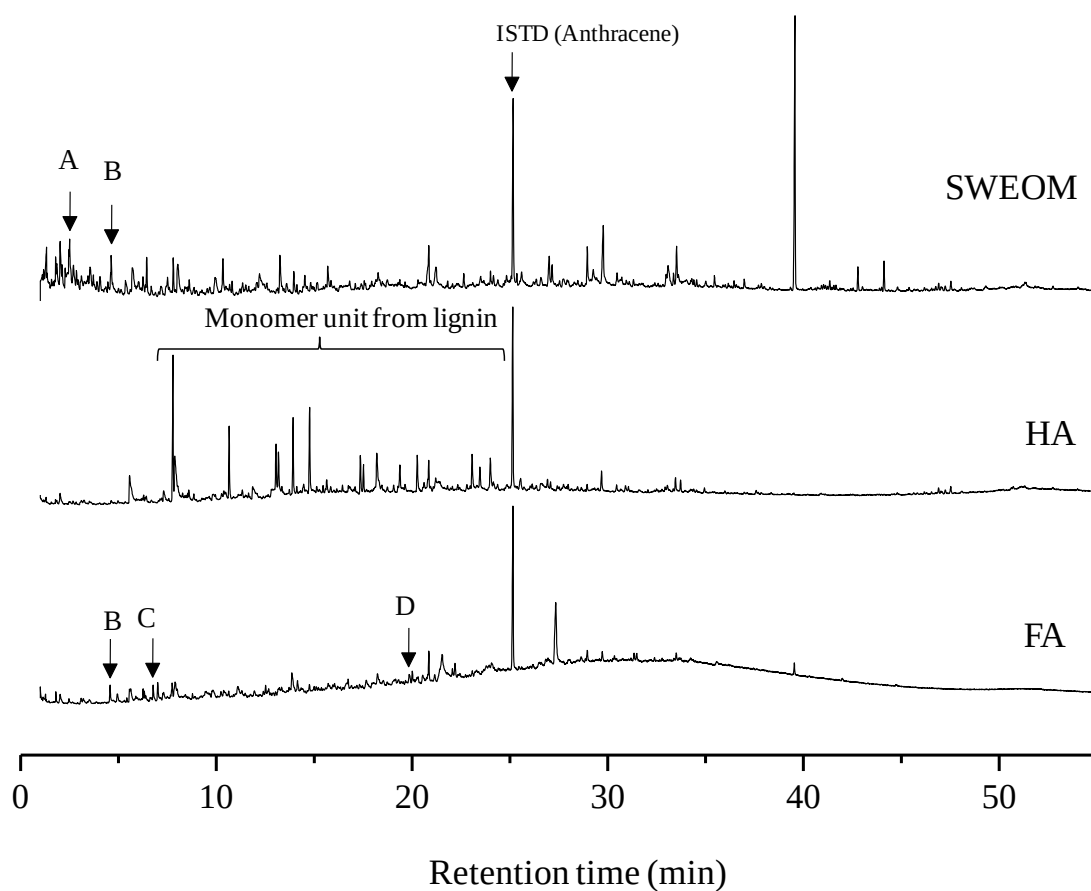


Fig. 2.6. Pyrograms by py-GC/MS for SWEOM, HA and FA. The labeled peaks (A – D) represent furan-derivatives; A: 2-furancarboxaldehyde, B: 5-methyl-2-furancarboxaldehyde, C: 3-methyl-2,5-furandione, D: 4,7-dimethyl-1,3-isobenzofurandione.

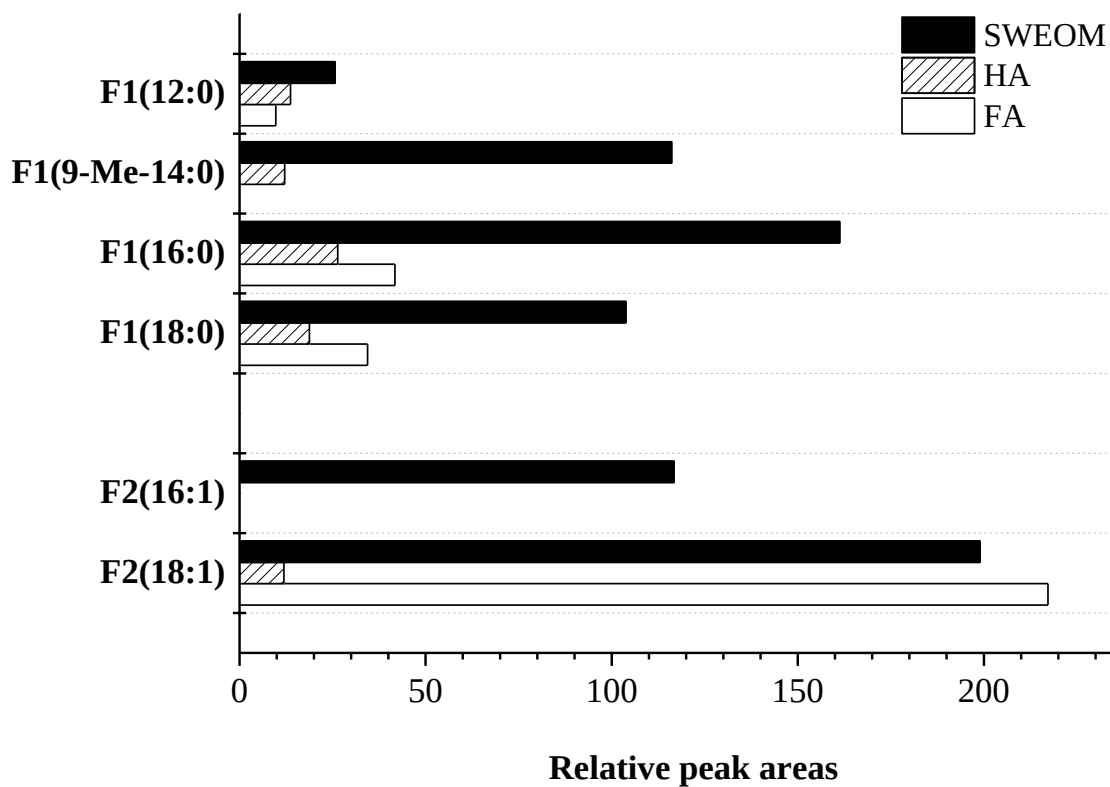


Fig. 2.7. Relative peak areas of remarkable identified fatty acid pyrolysate compounds for HA, FA and SWEOM. Each value is percentage of ISTD areas: (12:0) dodecanoic acid; (9-Me-14:0) 9-methyl-tetradecanoic acid; (16:0) hexadecanoic acid; (18:0) octadecanoic acid; (16:1) hexadecenoic acid; (18:1) octadecenoic acid.

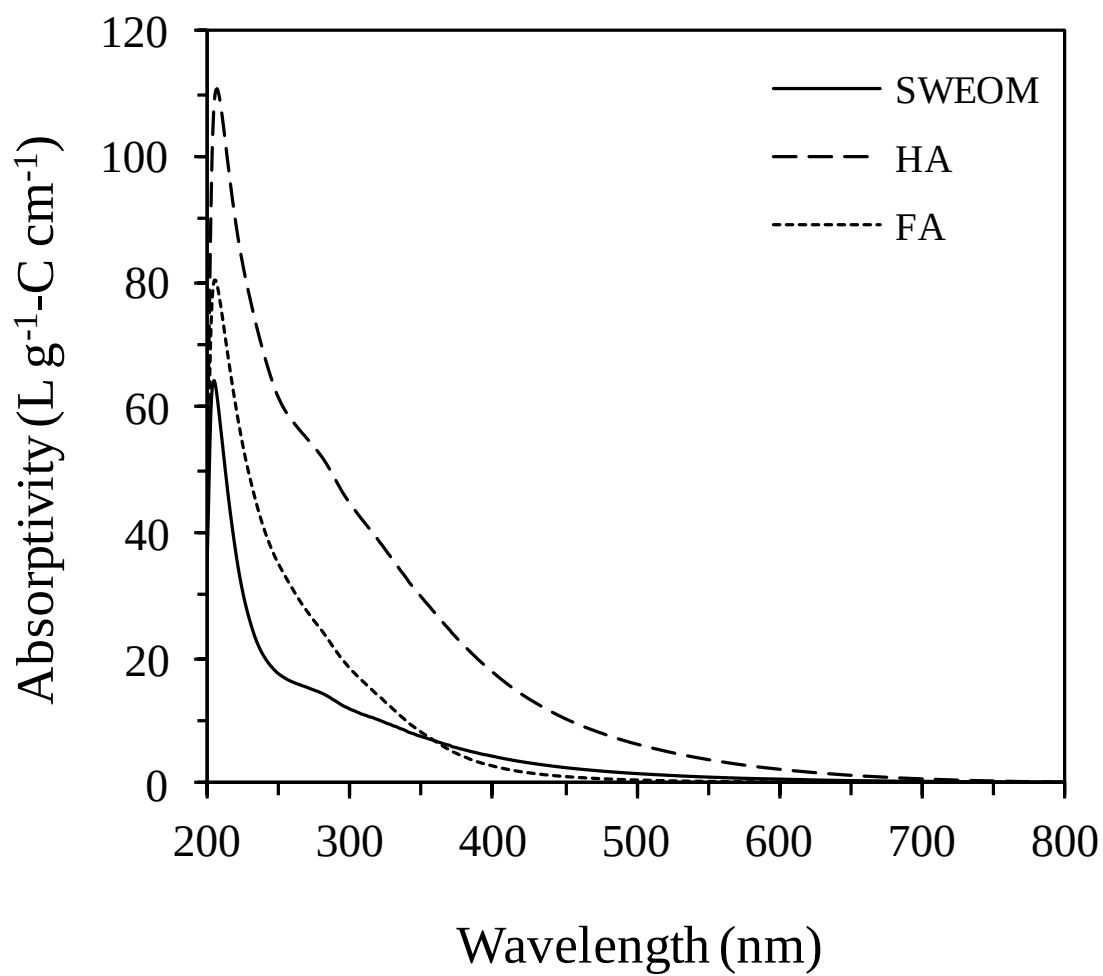


Fig. 2.8. UV-vis absorption spectra for HA, FA and SWEOM at pH 8, 20 mg L⁻¹ of sample concentrations.

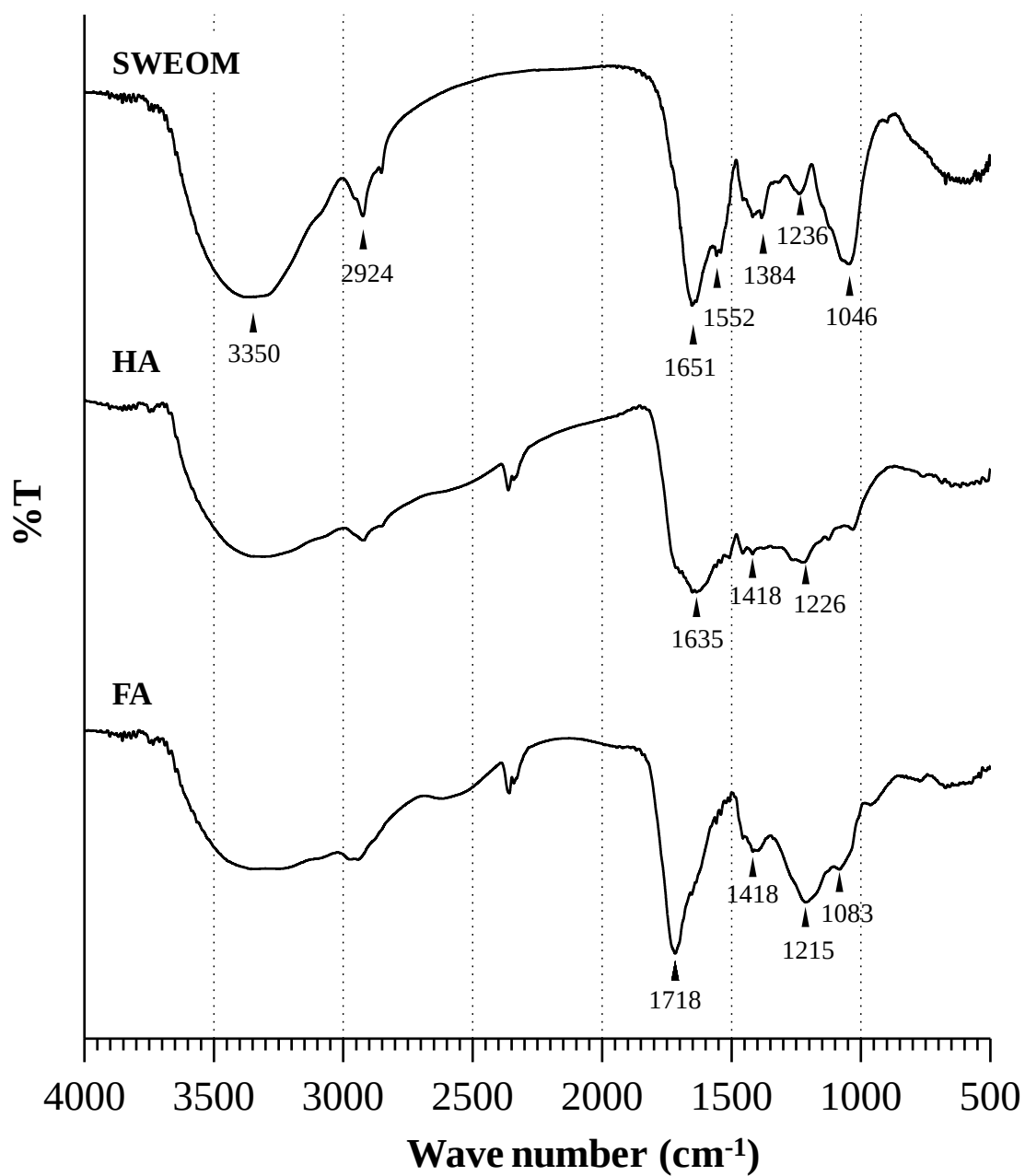


Fig. 2.9. FT-IR spectra for HA, FA and SWEOM with a KBr disk.

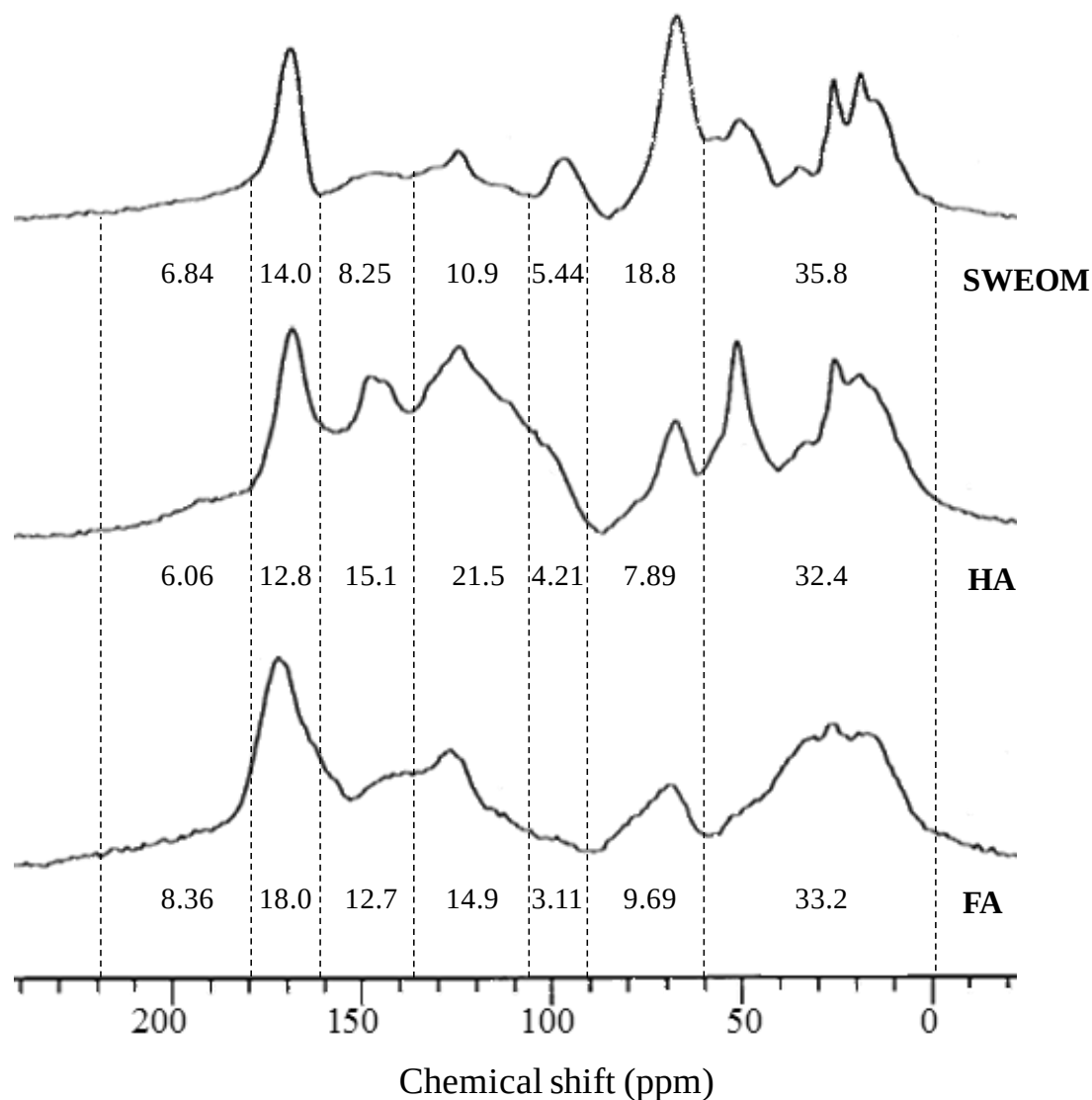


Fig. 2.10. Solid state CP-MAS ^{13}C NMR spectra for SWEOM, HA and FA. The labeled values indicate the compositions (%) of carbon species for each area corresponding to each chemical shift ranges. 0 – 60 ppm: alkyl carbons. 60 – 90 ppm: aliphatic alcohols and ethers carbons. 90 – 105 ppm: anomeric carbons. 105 – 135 ppm: aromatic carbons attached to proton or carbon. 135 – 160 ppm: aromatic carbons attached to oxygen. 160 – 180 ppm: carbonyl carbons (acids, esters and amides). 180 – 220 ppm: carbonyl carbons (quinones, ketones and aldehydes).

2.5. References in Chapter 2

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Chapter 3

Binding Capabilities and Dissociation Kinetics for Iron (II) Complexes with Seawater Extractable Organic Matter and Humic Substances in a Compost

3.1. Introduction

Areas with a lack of dissolved Fe species in coastal seawater are referred to as barren ground, a situation in which coastal areas are no longer able to maintain the growth of seaweed [1]. Thus, the fertilization of barren coastal ground with soluble Fe is an important technique, in terms of restoring seaweed-beds. In coastal seawater in the presence of oxygen, Fe species are converted into insoluble Fe(III)-hydroxides, which result in the Fe no longer being available as a nutrient for the growth of seaweed. Therefore, soluble Fe species in seawater are present in the form of complexes with dissolved organic matter (DOM), such as humic substances that contain humic (HA) and fulvic (FA) acids. As described in previous chapters, a steel-slag/compost fertilizer was developed to supply dissolved Fe complexed with DOM to barren coastal areas, and this resulted in the restoration of seaweed-beds. I focused on seawater extractable organic matter (SWEOM) fraction that can serve as the major DOM fraction from the compost. The structural features of SWEOM were addressed in Chapter 2. However, information related to the complexation of Fe with SWEOM from the compost does not appear to be readily available.

On the other hand, Fe(III)-oxides, such as hematite and magnetite, are found on the surface of the steel slag [2]. Such Fe(III)-oxides can be reduced to soluble species in the presence of DOM, such as humic substances [3]. DOM is capable of reducing Fe(III), and all of the reduced Fe(II) becomes complexed with DOM [4]. Thus, in the case of fertilization using the a steel slag/compost mixture, Fe(II)-DOM complex species would be expected to be the major Fe component. In terms of the stability of Fe(II)-DOM complexes, their dissociation kinetics as well as their well-known

complexing abilities, are important, because of their impact on the lifetime of such species in the environment. The dissociation kinetics of metal complexes with HA and FA have been evaluated, based on the alteration of complexation equilibria: e.g., pH changes[5], the addition of competing cations [6], and the removal of free metal species by chelation using resins or anodic stripping voltammetry [7,8]. *Ortho*-phenanthroline (OP) is a well-known colorimetric reagent that is used in the analysis of Fe(II) [9]. Because OP produces a color change only when complexing with free species of Fe(II), this has been used in speciation analysis and the evaluation of the binding abilities of HAs to Fe(II) [10]. If free Fe(II) would bind to OP in the presence of DOM, the Fe(II)-DOM complexes would undergo dissociation and the formation of Fe(II)-OP complexes would be increased via ligand-exchange reactions. In this chapter, Fe(II)-binding abilities and dissociation kinetics for Fe(II)-DOM complexes were evaluated by a colorimetric method using OP, and parameters for SWEOM were compared with the corresponding values for HA and FA fractions that were extracted from the same compost sample.

3.2. Materials and Methods

3.2.1. Materials

A matured bark compost (Mori Industry Co., Ltd., Hokkaido, Japan) was freeze-dried, and particles smaller than 2 mm diameter were used in the tests. Artificial seawater was prepared by dissolving the following salts (g) in 1 kg of ultrapure water: NaCl 28.5; MgSO₄·7H₂O 6.82; MgCl₂·6H₂O 5.16; CaCl₂ 1.11; KCl 0.725; SrCl₂·6H₂O 0.024; NaBr 0.084; H₃BO₃ 0.024. Prior to use in the test, the pH of the artificial

seawater was adjusted to 8.1 using dilute aqueous NaOH.

3.2.2. Extraction and purification of HA, FA and SWEOM

The SWEOM was extracted and purified, as described in section 2.2.4. To separate the SWEOM from a seawater matrix, ultrafiltration and dialysis were employed. The equilibration period for the extraction with seawater was preliminary checked by monitoring UV-vis absorption spectra and TOC, and 3 days of shaking were found to be sufficient. A mixture of dry compost and artificial seawater at pH 8.1 (solid/liquid = 1/10, w/w) was shaken under a N₂ atmosphere for 3 days. The suspension was then centrifuged at 10,000 rpm for 15 min, and the supernatant was filtered through a 5A filter paper (ADVANTEC). The filtrate was then deionized by ultrafiltration with an ultrafiltration membrane filter (molecular weight cut-off of 0.5 kDa, regenerated cellulose, Millipore). The retained fraction was washed with ultrapure water several times. The obtained fraction was then dialyzed against ultrapure water using a Spectra/Por[®] dialysis tube (molecular weight cut-off of 0.5 kDa). After the dialysis, the fraction in the dialysis tube was freeze-dried, and a powdered sample of SWEOM was obtained. HA and FA were extracted and purified according to IHSS method, as described in section 2.2.3. The mixture of dry compost and 0.05 M aqueous NaOH (solid/liquid = 1:10, w/w) was shaken under nitrogen atmosphere for 24 hours. HA and FA fractions in the extract were separated and purified, as described in previous chapter [11]. Structural features of SWEOM, HA and FA were summarized in Tables 2.2 and 2.3 in chapter 2.

3.2.3. Complexometric titration and dissociation kinetics

Flowsheet of methods for complexometric titration and analysis of dissociation kinetics are shown in Fig. 3.1. Stock solutions of DOM for SWEOM, HA and FA (1000 mg L^{-1}) were prepared by dissolving powdered samples in 0.05 M NaOH aqueous. A stock solution of Fe^{2+} (0.5 mM) was prepared by dissolving $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$ in a 0.02 M HCl aqueous. Buffer solutions ($\text{pH } 3.6$) were prepared by mixing 0.2 M aqueous acetic acid and sodium acetate. A $0 - 100 \text{ }\mu\text{L}$ aliquot of the Fe^{2+} stock solution was mixed with the buffer solution ($4.7 - 4.8 \text{ mL}$) including the DOM stock solution ($100 \text{ }\mu\text{L}$) in a 15 mL glass tube, and this was incubated at $25 \text{ }^\circ\text{C}$ for 24 hours in the dark. After adding a $100 \text{ }\mu\text{L}$ aliquot of aqueous OP (5 mM) and allowing the solution to stand for 30 min, the absorbance at 510 nm was measured using a V-630 type UV-vis spectrometer (Japan Spectroscopic Co., Ltd.).

A 4.8 mL aliquot of buffer solution including DOM (20 mg L^{-1}) was placed to a 10-mL glass tube, and a $200 \text{ }\mu\text{L}$ of aliquot of the Fe^{2+} stock solution was then added. This mixture was incubated for 24 hours at $10, 20, 30$ and $40 \text{ }^\circ\text{C}$. After adding a $100 \text{ }\mu\text{L}$ of OP (5 mM) and allowing the solution to stand for 30 min, the absorbance at 510 nm was measured. The initial concentration of Fe(II)-DOM complex was determined from the total and free concentrations of Fe(II) in that time. After 2-, 4-, 6- or 8-hours incubation periods at $10, 20, 30$ or $40 \text{ }^\circ\text{C}$, the absorbance values were measured at 510 nm , and the concentrations of the Fe(II)-DOM complexes calculated for each incubation period.

3.3. Results and Discussions

3.3.1. Binding abilities

In the presence of Fe^{2+} and DOM such as HA, FA and SWEOM, the OP binds to free Fe(II) , but not to Fe(II)-DOM complexes [10]. Such properties of OP can be applied to the complexometric titrations to evaluate the Fe(II) -binding abilities of DOM [10]. The colored species of Fe(II)-OP is a complex between Fe^{2+} and OP in a 1:3 molar ratio. The picture of colored Fe(II)-OP complex under the acetate buffer is shown in Fig. 3.2. In the optimized conditions, the 1:3 complexes are the dominant species [9]. Thus, Fe(II)-OP in this study denote 1:3 complexes between Fe(II) and OP. Assuming a 1:1 molar ratio for the complexation between Fe^{2+} and an arbitrary binding site in DOM (DOM_i), the conditional binding constant (K_b) can be defined as:

$$K_b = \frac{[\text{Fe(II)-DOM}_i]}{[\text{Fe}^{2+}][\text{DOM}_i]} \quad (1)$$

For the complexometric titration, the total Fe^{2+} concentration was varied from 0 to 10 μM at a constant concentration of DOM (20 mg L^{-1}), and $[\text{Fe}^{2+}]$ was colorimetrically determined using OP. Thus, the total concentration of complex species ($\Sigma[\text{Fe(II)-DOM}_i]$) can be calculated by subtracting $[\text{Fe}^{2+}]$ from the total concentration of Fe^{2+} . The total concentration of binding sites to Fe(II) in DOM (C_{DOM}) can be expressed as: $C_{\text{DOM}} = \Sigma[\text{DOM}_i] + \Sigma[\text{Fe(II)-DOM}_i]$. Combining this with Eq. (1), the relationship between $[\text{Fe}^{2+}]$ and $\Sigma[\text{Fe(II)-DOM}_i]$ can be derived as follow:

$$\Sigma[\text{Fe(II)-DOM}_i] = \frac{C_{\text{DOM}} K_b [\text{Fe}^{2+}]}{1 + K_b [\text{Fe}^{2+}]} \quad (2)$$

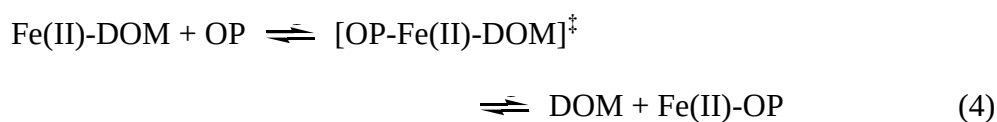
Plots and curves fitted by Eq. (2) are shown in Fig. 3.3. A non-linear least square regression analysis of the experimental data set for $[\text{Fe}^{2+}]$ and $\Sigma[\text{Fe(II)-DOM}_i]$ to Eq. (2) ($R^2 = 0.96 \sim 0.99$) resulted in estimated K_b and C_{DOM} values. The binding capacity of DOM to Fe(II) (BC) can be calculated as [12]:

$$BC = \frac{C_{\text{DOM}}}{[\text{DOM}(\text{g L}^{-1})] \times \%C} \times 100 \quad (3)$$

The estimated $\log K_b$ and BC values for the DOM are summarized in Table 3.1. In the present study, the acetate buffer was used to adjust the pH and this may affect the complexation equilibrium between Fe(II) and DOM. However, the stability constants for Fe(II)-acetate ($K = 0.54 - 1.40$) [13] were much smaller than those for Fe(II)-DOM complexes ($K = 1.0 \times 10^5 - 4.3 \times 10^5$), indicating that the influences of Fe(II)-acetate formation would be negligible. The $\log K_b$ values for SWEOM and FA were significantly larger than that for HA, suggesting that the stability of the complex produced between Fe(II) and SWEOM is comparable to that with FA. However, the BC value for SWEOM was significantly smaller than those for HA and FA, and this was consistent with the low value for the cation exchange capacity in SWEOM (Table 2.2 in chapter 2).

3.3.2. Dissociation kinetics and activation parameters

Fe(II)-DOM complexes can be dissociated in the presence of a strong chelator, such as OP. Dissociation of the Fe(II)-DOM complex may proceed *via* the formation of a ternary complex (OP-Fe(II)-DOM), analogous to that reported for metal-OP complexes [14]:



The dissociation kinetics of the Fe(II)-DOM complexes were monitored by the increase in the Fe(II)-OP complex content during the reaction (4). However, the pseudo-first-order rate constant (k_{obs}) was evaluated, based on a decrease of $\Sigma[\text{Fe(II)-DOM}_i]$, as described in the following equation:

$$\ln \frac{\Sigma[\text{Fe(II)}-\text{DOM}]_{t=t'}}{\Sigma[\text{Fe(II)}-\text{DOM}]_{t=0}} = -k_{\text{obs}} \times t \quad (5)$$

where t represents the incubation time after adding OP. The kinetic curves for the dissociation reaction are shown in Fig. 3.4. As shown in Fig. 3.4, Fe(II) complexed with SWEOM (●) was immediately dissociated, compared to those with HA (■) and FA (▲). However, the dissociation of Fe(II)-EDTA was not observed, even if the reaction mixture would stand for 24 h (★ in Fig. 3.4). This indicates that Fe(II) complexed with EDTA is not dissociable in the presence of the OP, because EDTA can strongly bind with Fe(II). In contrast, as shown in Fig. 3.5, the color of Fe(II)-OP solution was decolorized by adding EDTA to Fe(II)-OP solution. Approximately 3 % of total [Fe(II)-OP] was dissociated after 72 hours in the presence of EDTA. This reaction was very slow, however, Fig 3.5 indicates Fe(II) complexed with OP can be dissociated in the presence of strong chelator such as EDTA. In reaction (4), the tentative species, $[\text{OP-Fe(II)-DOM}]^\ddagger$, can be regarded as an activated complex that is produced during the dissociation of Fe(II)-DOM to form Fe(II)-OP *via* a ligand-exchange. To evaluate the activation parameters for the reaction (4), the temperature dependence on k_{obs} was investigated in the range of 10 – 40 °C. The free energy of activation, $\Delta G^\ddagger$, can be expressed as [4]:

$$k_{\text{obs}} = \chi \frac{k_{\text{B}}T}{h} \exp\left(-\frac{\Delta G^\ddagger}{RT}\right) \quad (6)$$

where h , k_{B} , T and R are Planck's constant, the Boltzmann constant, the absolute temperature and the universal gas constant, respectively. χ is transmission coefficient (0.5 ~ 1.0), which represents a degree of dissociation of DOM in the activated complex. From Eq. (6), $\Delta G^\ddagger$ can be evaluated using k_{obs} at an arbitrary temperature. Since $\Delta G^\ddagger =$

$\Delta H^\ddagger - T\Delta S^\ddagger$, Eq. (6) can be rearranged by using the activation enthalpy ($\Delta H^\ddagger$) and entropy ($\Delta S^\ddagger$),

$$\ln \frac{k_{\text{obs}}}{T} = \ln \chi \frac{k_B}{h} + \frac{\Delta S^\ddagger}{R} - \frac{\Delta H^\ddagger}{R} \times \frac{1}{T} \quad (7)$$

From the linear relationships between $\ln (k_{\text{obs}}/T)$ and $1/T$ for each Fe(II)-DOM complex (Eyring plots), $\Delta S^\ddagger$ and $\Delta H^\ddagger$ were evaluated. Eyring plots are shown in Fig. 3.6. The evaluated $\Delta G^\ddagger$ (283 K), $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are summarized in Table 3.1. Activation parameters were calculated, where $\chi = 1$ (i.e., $\ln (\chi \cdot k_{\text{obs}}/T) = 23.76$). In general, $\Delta S^\ddagger$ provides information on the stability of activated complex species [15]. The levels of $\Delta G^\ddagger$ and $\Delta S^\ddagger$ were similar for Fe(II) complexes with HA, FA and SWEOM. These results suggest that the reaction intermediate for the dissociation of Fe(II)-DOM complex *via* a ligand-exchange with OP is the ternary complex, OP-Fe(II)-DOM, and its stability is similar for the HA, FA and SWEOM ligands. Some researchers have mentioned that free or dissociated metal species are more bioavailable than complex species with DOM [16-18]. Because $\Delta S^\ddagger$ values for HSs and SWEOM indicated constant ($-236 \sim -267 \text{ KJ mol}^{-1}$, Table 3.1) that ligand-exchange reaction would be a major process for Fe-uptake by marine biota, in which complex species are dominant. However, the $\Delta H^\ddagger$ value for the Fe(II)-SWEOM complex was significantly smaller than those for Fe(II) complexes with HA and FA (Table 3.1, slope of fitting lines in Fig. 3.6). $\Delta H^\ddagger$ can be regarded as the energy for dissociation of the activated complex, in which the smaller $\Delta H^\ddagger$, the more dissociable is the complex. Therefore, the smaller $\Delta H^\ddagger$ for SWEOM indicates that the Fe(II)-SWEOM complex is more dissociable and exchangeable than Fe(II) complexes with HA and FA.

3.4. Conclusion

For a restoration technique using fertilizer comprised of steel slag and compost, SWEOM can be attracted as a novel chelator of Fe(II) in coastal seawater. This chapter proved that SWEOM can complexed with Fe(II). The Fe(II) binding capacity of SWEOM as smaller than those for HA and FA. However, the smaller $\Delta H^\ddagger$ of Fe(II)-SWEOM suggest that Fe(II) bound with SWEOM can easily dissociate, compared to HA and FA. Strictly speaking, the experimental conditions in this experiment (pH 3.6, $I = 0.02$) were remarkably different from those in seawater (pH 8.1, $I = 0.7$). Thus, to understand a role of SWEOM in the restoration technique, the binding abilities under seawater conditions and bioavailability to algae should further be addressed.

Table 3.1. Binding characteristics, dissociation rates and activation parameters^α for Fe(II) complexes with SWEOM, HA and FA.

Sample	Binding ability		Dissociation rate ^β	Activation parameter		
	$\log K_b$	BC ($\mu\text{mol g}^{-1}\text{C}$)	k_1 ($\times 10^{-5} \text{ s}^{-1}$)	$\Delta H^\ddagger$ (kJ mol^{-1})	$\Delta S^\ddagger$ ($\text{J K}^{-1} \text{ mol}^{-1}$)	$\Delta G^{\ddagger\gamma}$ (kJ mol^{-1})
SWEOM	5.63 $\pm$ 0.15	80.0 $\pm$ 7.9	2.91 $\pm$ 0.73	18.8 $\pm$ 1.1	- 267 $\pm$ 4	94.3 $\pm$ 2.2
HA	5.01 $\pm$ 0.26	235 $\pm$ 62	1.85 $\pm$ 0.61	26.6 $\pm$ 3.4	- 243 $\pm$ 11	95.5 $\pm$ 6.6
FA	5.39 $\pm$ 0.10	112 $\pm$ 10	2.75 $\pm$ 0.39	27.0 $\pm$ 1.6	- 236 $\pm$ 5	93.8 $\pm$ 3.1

^α Each analysis was conducted 4 – 10 times. “ $\pm$ ” represents standard deviation.

^β Dissociation rate represents pseudo-first-order reaction rate constant.

^γ $\Delta G^\ddagger$ values were evaluated at 283 K.

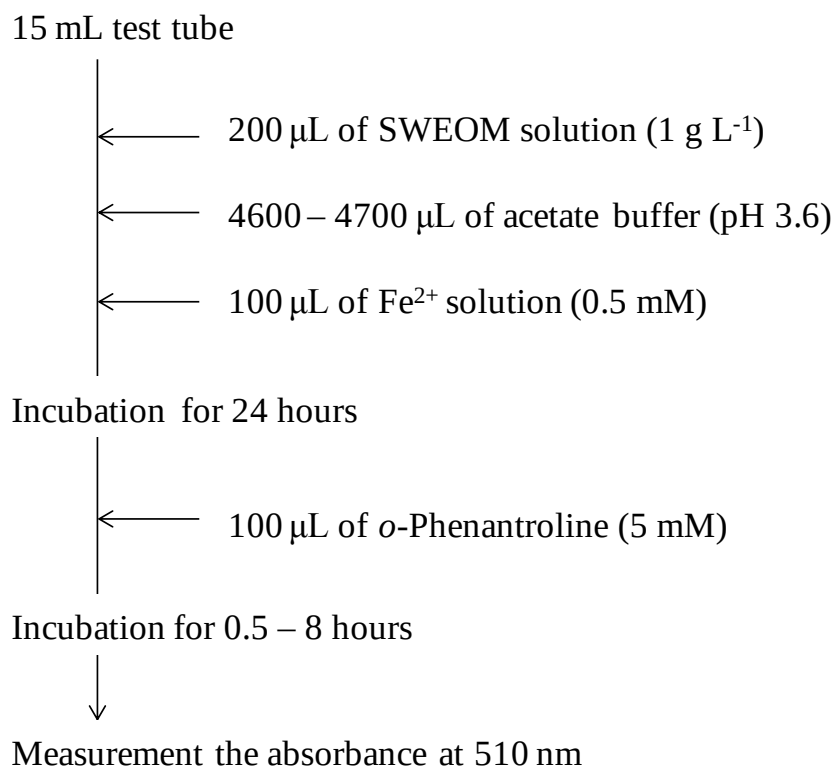


Fig. 3.1. Flowsheet of complexometric titration using *ortho*-phenantroline.

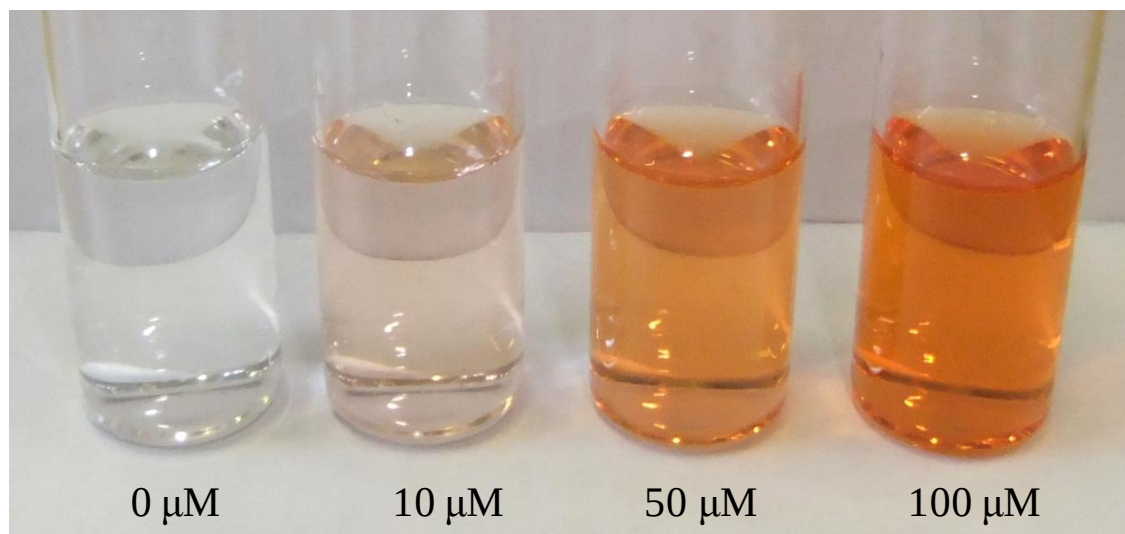


Fig. 3.2. Colors of Fe(II)-OP complexes at pH 3.6 with acetate buffer. Labeled values represent total [Fe] (μM). Total [OP] is 1 mM.

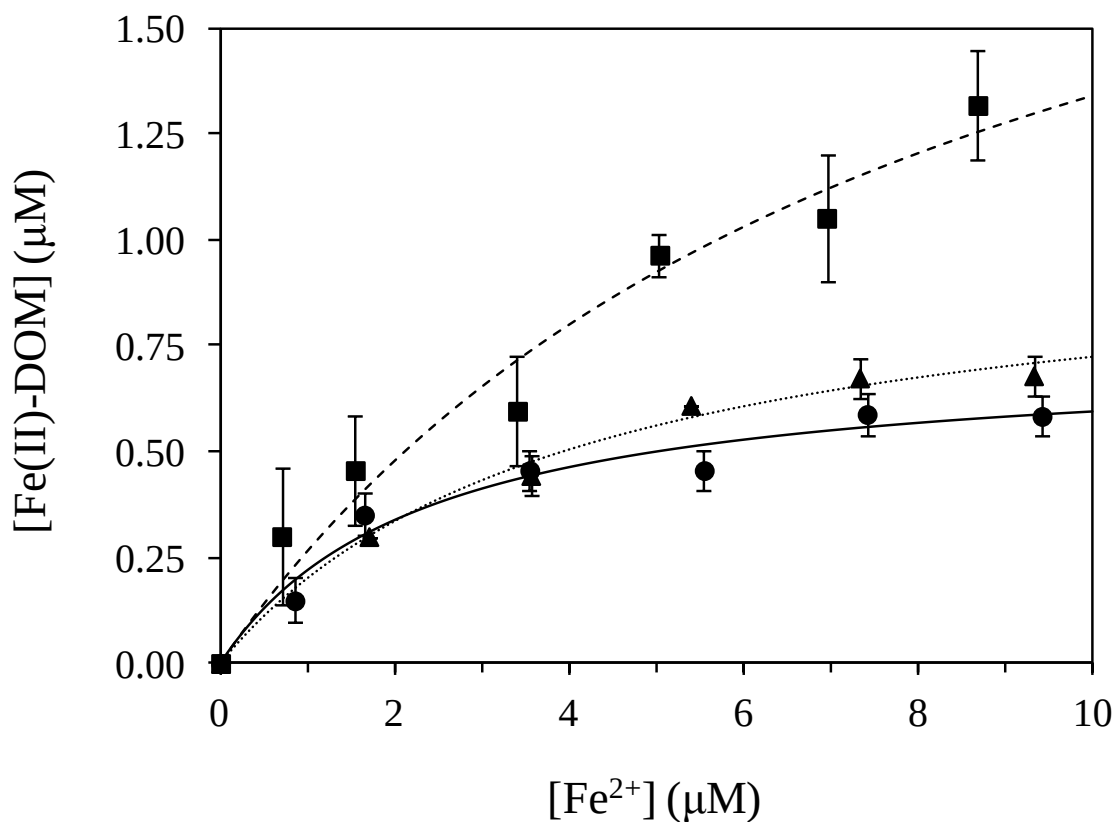


Fig. 3.3. Complexometric titration curves for Fe(II). Circle plots and solid line, square plots and dashed line, and triangle plots and dotted line represent experimental plots and curves fitted by eq. (2) for SWEOM, HA and FA, respectively. Concentrations of SWEOM, HA and FA were 20 mg L^{-1} , OP was 100 μM . Error bars represent standard deviation ($n = 3 \sim 8$).

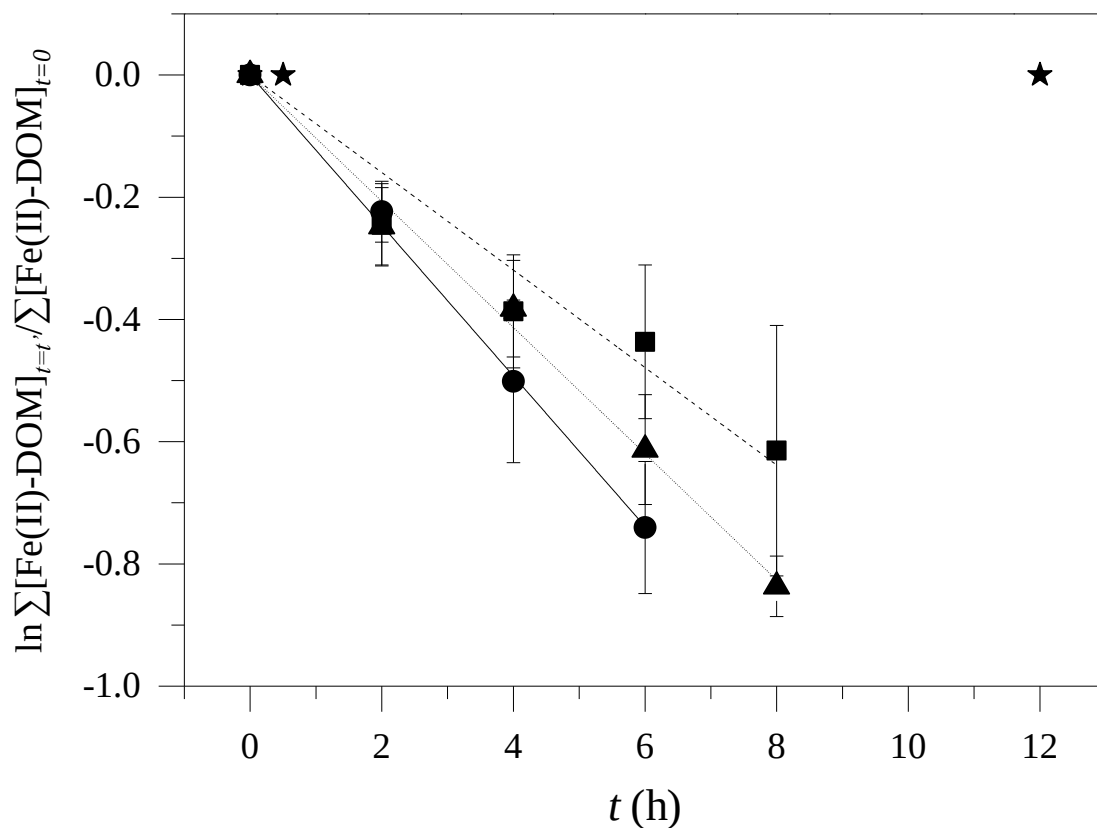


Fig. 3.4. Dissociation kinetics for the Fe(II)-DOM complexes at 283 K. Circle plots and solid line, square plots and dashed line, and triangle plots and dotted line represent experimental plots and curves fitted by eq. (5) for SWEOM, HA, and FA, respectively. Star plots represent the case for EDTA. In the case for SWEOM, HA and FA, [DOM], total [Fe(II)] and [OP] were 40 mg L⁻¹, 10 μM and 100 μM, respectively. For EDTA, [EDTA], total [Fe(II)] and [OP] were 50, 50 and 500 μM, respectively. Error bars represent standard deviation ($n = 4$).

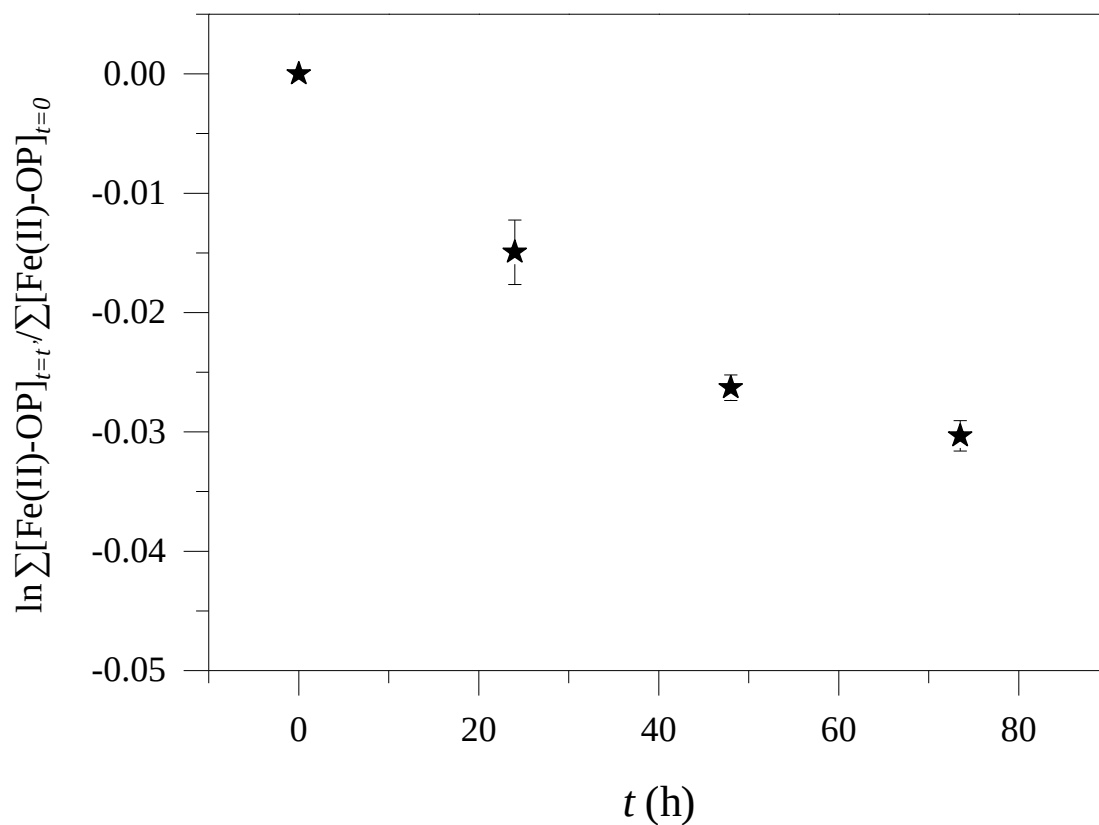


Fig. 3.5. Dissociation kinetics for the Fe(II)-OP complexes under the presence of EDTA at 298 K. [EDTA], total [Fe(II)] and [OP] were 50, 50 and 500 μM , respectively. The data of the absence of EDTA were used as calibration. Error bars represent standard deviation ($n = 3$).

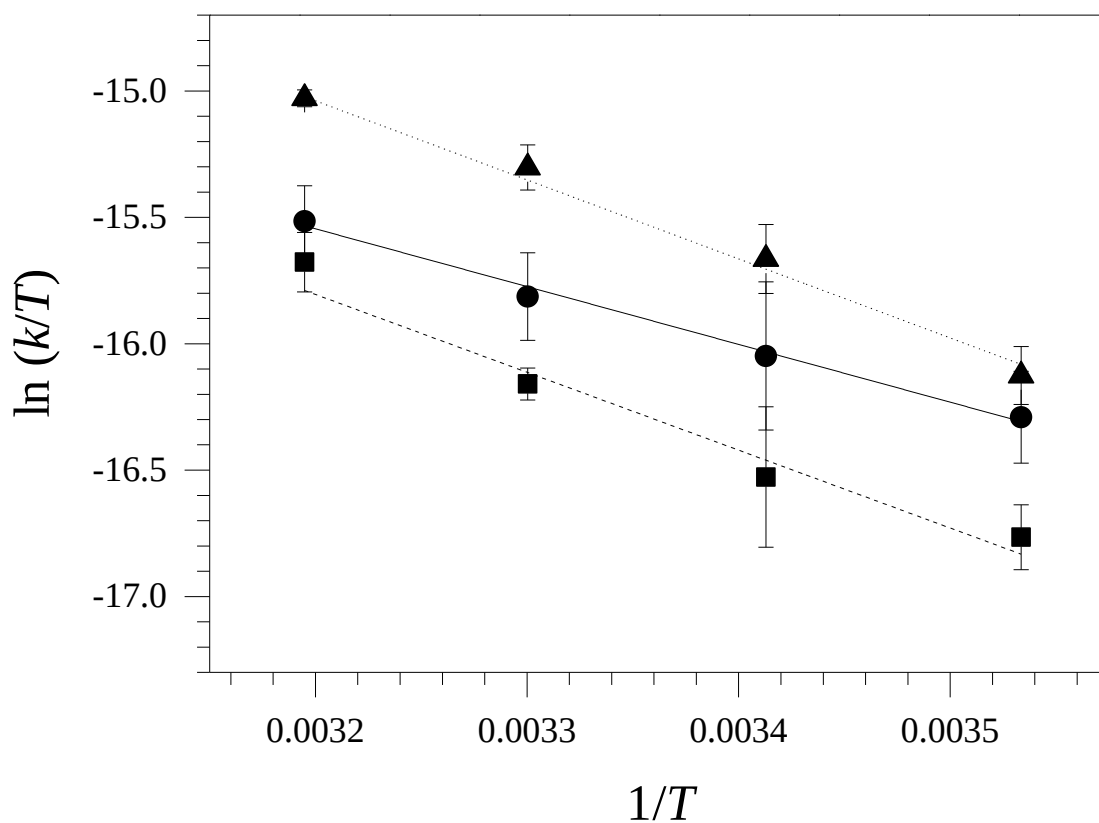


Fig. 3.6. Eyring plots for dissociation of Fe(II)-DOM complexes. Circle plots and solid line, square plots and dashed line, and triangle plots and dotted line represent experimental plots and curves fitted by eq. (7) for SWEOM, HA and FA, respectively. Error bars represent standard deviation ($n = 3 \sim 8$).

3.5. References in Chapter 3

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Chapter 4

***Determination of Labile Fe(II) Complexes
with Seawater Extractable Organic matter
under Seawater Conditions Based on the
Kinetics of Ligand-exchange Reactions with
Ferrozine***

4.1. Introduction

As described in section 1.4, barren ground is a phenomenon associated with the depletion of seaweed in coastal areas. The development of barren ground has been attributed to be a lack of soluble Fe ($< 1 \text{ nM}$) [1], which is an essential micronutrient for the growth of algae [2,3]. In macroalgae, the uptake of soluble iron is required for the gametophyte to produce an oogonium or an antheridium [4-7], and a sporophyte is formed from the matured gametophyte [3]. Thus, a lack of dissolved Fe in coastal areas seawater can lead to seaweed depletion. It is known that dissolved organic matter plays an important role in the mobility, solubility and bioavailability of trace metals in terrestrial and aquatic environments [2,8-12]. In seawater, the majority of dissolved ferric (Fe(III)) and ferrous (Fe(II)) species are present in the form of complexes with dissolved organic matter [2, 13-15]. Based on this, a fertilizer comprised of a steel slag and compost was tested for its ability to supply dissolved Fe to barren coastal areas, and this attempt was successful and resulted in the restoration of seaweed-beds [16]. In this technique, seawater extractable organic matter (SWEOM) from the compost serves as a chelator of Fe and permit it elute from the steel slag [17,18]. Fe(III)-oxides are found on the surface of the steel slag [19], and can be reduced to soluble Fe(II) species in the presence of dissolved organic matter [20]. It has been reported that all of the reduced Fe(II) species are complexed with dissolved organic matter [21]. Therefore, the nature of the complex produced between SWEOM and Fe(II) need to be evaluated to better understand the performance of such fertilizers.

On the other hand, estimating the bioavailability of a metal to aquatic biota is an important approach [22,23]. The kinetic stability of Fe(II)-SWEOM complexes is a key

factor in determining the bioavailability of Fe(II) species [2,3,22,24]. Readily dissociable complexes (labile species) are the likely primary source of soluble iron for algae [2,19,25-27]. A key factor in the uptake of Fe(II) involves ligand-exchange reactions between dissolved organic matter and receptor proteins on the cell-membrane [2]. The kinetics of ligand-exchange reactions of Fe(II)-humic acid complexes have been investigated using *ortho*-phenanthroline or ferrozine (FZ) as models of receptor proteins (details are shown in Chapter 3 or [17,28]), although the conditions (pH 3.6 or 5, $I = 0.02$) were far from those for seawaters (pH 8, $I = 0.7$). To investigate the contribution of SWEOM as a chelator of Fe, it should be examined that the complexation ability under the conditions of seawater. However, it is difficult to determine both free and complex species of Fe(II) at higher pH and ionic strength, because Fe(II) is readily oxidized to insoluble Fe(III)-hydroxides, which precipitate from the solution [29]. Indeed, pH and ionic strength both have a dramatic effect on the speciation of dissolved metal ions in the presence of DOM, such as humic acids [11,30-34]. Therefore, the determination of labile species of Fe(II)-SWEOM complexes under seawater conditions (pH 8, $I = 0.7$) is needed to realistically evaluate contributions of the fertilizer to their bioavailability. FZ colorimetry has been employed for the analysis of Fe(II) in seawater after reducing the total iron to ferrous forms and adjusting the pH to 4 – 6 [35,36]. However, the determination of labile complex species of Fe(II) that are complexed with dissolved organic matter, such as SWEOM, using FZ has not been examined under seawater conditions. In this chapter, labile species of Fe(II)-SWEOM complexes were determined, based on the difference in the reaction kinetics between free Fe(II) complexed with FZ and a ligand-exchange of Fe(II)-SWEOM complexes involving FZ. In addition, this method was utilized to

evaluate the conditional binding constants and binding capacities for the formation of labile complexes of Fe(II) with molecular weight fractionated SWEOM samples.

4.2. Materials and methods

4.2.1. Materials

Ammonium iron(II) sulfate hexahydrate ($\text{Fe}(\text{SO}_4)_2(\text{NH}_4)_2 \cdot 6\text{H}_2\text{O}$), tris(hydroxymethyl)aminomethane, hydroxylamine, D(+)-glucose and L(+)-glutamic acid were purchased as special reagent grade from Nacalai Tesque (Kyoto, Japan). L(+)-Ascorbic acid was purchased as special reagent grade from Wako Pure Chemical Industries (Osaka, Japan). 4,4'-[3-(2-Pyridyl)1,2,4-triazine-5,6-diyl]bis(benzene sulfonic acid) disodium salt hydrate (ferrozine, FZ) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Ultrapure water was prepared by purifying deionized, distilled water using a Millipore Simplicity[®] UV system. The compost sample, prepared by maturing the mixture of bark-tips and cowpat, was obtained from Mori Industry Co., Ltd. (Hokkaido, Japan). The compost sample was freeze-dried and passed through a 2 mm mesh stainless-steel sieve. Compost particles less than 2 mm in size were used in the tests. The elemental compositions of the compost sample were as follows: C 38.8; H 4.86; N 2.45; S 0.43; ash 18.0 (wt %).

4.2.2. Fractionation of SWEOM samples

Flowsheet of methods for the extraction and molecular weight fractionation of SWEOMs are shown in Fig. 4.1. SWEOM was extracted with artificial seawater from the bark compost, according to previous studies (Chapter 2 and [17,18]). A mixture of

compost and seawater (solid/liquid = 1:10, wt/wt) was shaken under a N₂ atmosphere for 3 days. The suspension was centrifuged, and the supernatant was then filtered through an ADVANTEC 5A filter paper. The filtrate was fractionated using regenerated-cellulose ultrafiltration membranes (molecular weight cut-off 100, 30, 10, 5 and 0.5 kDa, Millipore), and following fractions were obtained: > 100 kDa, 30-100 kDa, 10-30 kDa, 5-10 kDa, 0.5-5 kDa and < 0.5 kDa. The obtained fractions were purified by dialysis against ultrapure water (molecular weight cut-off 0.5 kDa dialysis tube). The dialyzed fraction was freeze-dried to give powdered SWEOM samples. The fraction with a molecular weight below 0.5 kDa was acidified to pH < 1.0 with concentrated HCl. The acidified fraction was passed through a DAX-8 resin, Which is an acrylic-ester resin for the adsorbent of phenolic moieties in humic matter [37]. The adsorbed fraction was then eluted with aqueous 0.01 M NaOH, and the eluent was passed through an H⁺-type cation-exchange resin column (DOWEX[®] HCR-W2). Finally, a powdered sample was obtained by freeze-drying. Elemental compositions (%C, %H, %S and %Ash), amino acid contents and the acidic functional group content (carboxylic acids and phenolic hydroxyl groups) were determined, according to the procedures and conditions reported in previous reports [18,38]. To estimate the molecular size distribution, concentrations of total organic carbon (TOC) of collected fraction were analyzed by means of a TOC-V CSH analyzer (Shimadzu).

4.2.3. Analysis of labile Fe(II) species

A buffered seawater was prepared by dissolving following salts in 1 L of ultrapure water: NaCl 28.0; MgSO₄·7H₂O 7.0; MgCl₂·6H₂O 4.0; CaCl₂ 1.11; KCl 0.7; tris(hydroxymethyl)aminomethane 1.2; ascorbic acid 1.76 (g). The composition of the

buffered seawater was close to the values of the ASP₁₂ medium that is typically used for culturing marine macroalgae [4,5,39]. Ascorbic acid, hydroxylamine and glucose were tested as antioxidants. Stock solutions of SWEOMs (1000 mg L⁻¹) were prepared by dissolving powdered samples in 0.05 M aqueous NaOH solution. The stock solution of Fe²⁺ (2 mM) was prepared by dissolving Fe(SO₄)₂(NH₄)₂•6H₂O in 0.01 M aqueous HCl. The 50 mM FZ solution was prepared by dissolving it in ultrapure water. The absorbance values at 562 nm for the color of Fe(II)-FZ complex in the presence of ascorbic acid (10 mM) were independent of the solution pH (pH 3.6 ~ 8.3). Prior to use, the pH of the buffered seawater that contained 50 mg L⁻¹ of SWEOM was adjusted to 8.05 ± 0.05 with diluted aqueous NaOH or HCl. Aliquots of 0 - 70 µL of the 2 mM Fe²⁺ stock solution were added to the 4880 - 4950 µL aliquots of the buffered seawater and the solution were placed in 15 mL glass tubes. After a 10 min incubation, 2970 µL aliquots of this sample solution was placed in a 1 × 1 cm quartz cell. Subsequently, 30 µL of 50 mM aqueous FZ was added to the cell, and the increase in the absorbance at 562 nm was monitored at 20 °C with stirring until a plateau was reached. The absorbance measurements were performed using a V-630 type UV-vis spectrophotometer connected to a PAC-743 type temperature controller with a Peltier device (Japan Spectroscopic Co., Ltd.). Absorbance readings were collected at intervals of 1 s. In addition, kinetic curves for formation of Fe(II)-FZ complex were collected using an RSP-1000-02 type stopped-flow spectroscopy system (UNISOKU, Co. Ltd).

4.3. Results and Discussions

4.3.1. Colorimetric detection of labile Fe(II) complexes with SWEOM

Assuming a 1:1 molar ratio complexation between Fe(II) and an arbitrary binding site in SWEOM, the following equilibrium can be written:



After adding FZ to the equilibrium system of Eq. (1), the free species of Fe(II) would be immediately complexed with FZ, and a ligand-exchange reaction between SWEOM and FZ would then gradually occur, as follows:



In Eq. (3), the Fe(II)-SWEOM complex can be regarded as a labile Fe(II) species complexed with SWEOM (Fe(II)-SWEOM_{labile}). Although the reaction described by Eq. (2) is very fast, the ligand-exchange reaction in Eq. (3) is relatively slow [17]. However, the determination of Fe(II) species in the buffered seawater (pH 8) was difficult because of the facile auto-oxidation of Fe(II) to Fe(III) (□ in Fig. 4.2 and 4.3 (A)). This makes it impossible to discriminate between the free and complex species of Fe(II) complexed with SWEOM. To suppress the auto-oxidation of Fe(II), the addition of some anti-oxidants was examined. Figure 4.4 shows the influence of anti-oxidant concentration on the percentage of preserved Fe(II). Although hydroxylamine and glucose were not effective in preserving Fe(II) oxidation under seawater conditions, ascorbic acid was found to be a very effective antioxidant. Ascorbic acid has been employed as a reducing agent when determining total Fe in seawater in previous study [36]. As shown in Fig. 4.2 (■) and 4.3 (B), the detectable Fe(II) species were preserved by ascorbic acid, even in seawater conditions. In addition, the percentage of preserved Fe(II) reached plateau at ascorbic acid concentration above 10 mM (Fig. 4.4). Approximately 30% of Fe was not detected as Fe(II), even in the higher concentration

condition of ascorbic acid (Fig. 4.4). However, 90 ~ 95% of total Fe was detected as Fe(II)-ferrozine colored complex after starting for 24 hours. These results suggested that approximately 30% of Fe, a part of Fe in the seawater containing ascorbic acid exists as Fe(III) during the measurement of kinetic curves in Fig. 4.4. Because the decrease of pH was suppressed to within 0.1 pH unit during the analysis, we selected an ascorbic acid concentration of 10 mM for the evaluation of Fe(II)-binding abilities. The presence of ascorbic acid had no effect on the reaction kinetics due to the lower stability constant of Fe(II)-ascorbate ($\log K = -2.11 - -1.16$) [40]. At 50 μM Fe(II), the formed Fe(II)-ascorbate was estimated to be 0.008 – 0.07 % of total Fe(II) in the presence of 10 mM ascorbic acid, supporting the conclusion that the complexation of Fe(II) with ascorbic acid is negligible.

Figure 4.5 shows the kinetic curves for formation of the colored Fe(II)-FZ complex in the absence and presence of SWEOM. In the absence of SWEOM (Fig. 4.5, ○), the absorbance at 562 nm increased rapidly and reached a plateau within several sec. Using stopped-flow spectrophotometry, reaction kinetics of Fe(II) complexation with FZ in Eq. (2) in the absence or presence of SWEOM were monitored (Fig. 4.6, (A) or (C)). However, the gradual increases of optical density for the Fe(II)-FZ color in the presence of SWEOM were not detectable in the several-ten ms order by the kinetic curves from the stopped-flow spectrophotometry (Fig. 4.6 (B) and (C)). From the kinetic curves in Fig. 4.6 (A), the coloration by complexation between free Fe(II) and FZ was much faster than that by a reaction of Eq. (3), and a reaction of Eq. (2) was reached to a plateau within 0.03 s. Thus, due to a operating reason, the absorbance at 3s (Fig. 4.5) was employed as a period of the coloration of Fe(II)-FZ formation in the absence of ligand by Eq. (2). The absorbance values for the plateau region were

proportional to the concentrations of the initial Fe(II), indicating that the absorbance values in the absence of SWEOM can be used for purpose of calibration. However, in the presence of SWEOM (Fig. 4.5, ●, ■ and ▲), color formation was retarded compared to that in the absence of SWEOM. In addition, the degree of the retardation of the coloration increased with increasing SWEOM concentration. It is generally assumed that humic substances contain strong and weak binding sites [27,32,41-43]. The strong sites in SWEOM would be occupied first, with the formation of kinetically stable complexes [27,41]. As a consequence, large amounts of stable Fe(II)-SWEOM complexes were formed at higher concentrations of SWEOM. Thus, the retardation of coloring for the Fe-FZ complex in the presence of SWEOM can be attributed to free Fe(II) species and labile Fe(II)-SWEOM complexes which are ligand-exchangeable Fe(II) species to FZ.

SWEOM samples may contain the trace amounts of iron and this may affect the kinetic curves in Fig. 4.5. Thus, iron in SWEOM samples were determined by the FZ colorimetry after standing of the solution with ascorbic acid for 900 s. It was found that NMW, HMW and LMW contained 18, 36 and 8 $\mu\text{mol g}^{-1}$ of Fe, respectively. The kinetic curve for SWEOM (Fig. 4.5, ◆) showed that the effect of initial iron in the original sample of SWEOM is negligible.

As shown in Fig. 4.5, the absorbance value at up to 3 s was considered to represent the region for the complexation of free Fe(II) with FZ (Eq. 2) and corresponds to the concentration of the free species of Fe(II) for the kinetic curve in the presence of SWEOM. For the case of 50 mg L^{-1} of SWEOM (● in Fig. 4.5), subtracting the absorbance value at 3 s from that for the plateau region represents the concentration of labile Fe(II)-SWEOM complex species. Reaching the plateau of the kinetic curve in the

presence of SWEOM was determined to be the point when the variation in the absorbance for a 10 s incubation period was within 0.001.

4.3.2. Complexing abilities of SWEOMs to form labile complexes with Fe(II)

4.3.2.1. Molecular weight fractionated SWEOM samples

The SWEOM from the compost has a wide molecular weight distribution [17,18]. Because of this, it was fractionated, based on molecular size using an ultrafiltration technique. In addition, the complex species of Fe with SWEOM can be varied in molecular weight fraction of SWEOM [41]. Accordingly, the complexing abilities of size fractionated SWEOMs should be examined. To determine the TOC, fractions with molecular weights above 0.5 kDa (> 100 kDa, 100-30 kDa, 30-10 kDa, 10-5 kDa) were prepared by diluting the collected residue on the ultrafilter membrane to 100 mL with ultrapure water. The TOC value for the fraction below 0.5 kDa (TOC_{0.5 kDa<}) was estimated as a relative value based on the yield:

$$\text{TOC}_{0.5\text{kDa}<} = \text{TOC}_{>100\text{kDa}} \times \frac{\text{Yield}_{0.5\text{kDa}<}}{\text{Yield}_{>100\text{kDa}}} \quad (4)$$

where the yields for each fraction were calculated by dividing the mass of the obtained powdered fraction (g) by the mass of the initially obtained compost (kg). As shown in Fig. 4.7, the major fractions of the SWEOM had molecular weight fractions of > 100 kDa and < 0.5 kDa. The complexing abilities for forming labile species of the Fe(II)-SWEOM complex were evaluated for the following three fractions: > 100 kDa (HMW), > 0.5 kDa (NMW) and < 0.5 kDa (LMW). Among these fractions, the NMW fraction was the previously defined SWEOM, in which the structural features of this organic matter were compared with those for humic substances from the same origin

[18].

The elemental compositions and acidic functional group contents for NMW, HMW and LMW are summarized in Table 4.1. The elemental compositions of NMW and HMW were similar supporting the results shown in Fig. 4.7 that HMW is the major fraction in NMW. The H/C atomic ratio for LMW was significantly lower than the corresponding values for NMW and HMW, suggesting that higher amounts of unsaturation, such as aromatic functional groups, are present in this fraction. Although there were no significant differences in the O/C atomic ratio for all samples, LMW contained a higher amount of acidic functional groups, especially carboxylic acid groups, compared to those for NMW and HMW (Table 4.1). These results indicate that alcohols, ethers and esters are the major oxygen-containing functional groups in NMW and HMW. To compare the compositions of amino acids, the percentages of amino acids and total amino acids contents for NMW and LMW are summarized in Table 4.2. Although the N/C atomic ratio for LMW was lower than those for NMW and HMW, the total amino acid contents of LMW (28.2 mg g⁻¹-Sample) was much higher than that of NMW (15.3 mg g⁻¹-Sample). In particular, aspartic acid and glutamic acid in LMW were higher than those in NMW. These results suggest that LMW fraction is rich in protein components.

The NMR spectra and the compositions of carbon species for the NMW, HMW and LMW, which were calculated from the peak integration values in the solid-state CP-MAS ¹³C NMR spectra, are shown in Fig. 4.8. The peaks were assigned, based on the previous reports (Chapter 2, [18,44]): alkyl carbons (0 – 60 ppm); aliphatic alcohols and ethers carbons (60 – 90 ppm); anomeric carbons (90 – 105 ppm); aromatic carbons (105 – 160 ppm); carbonyl carbons (acids, esters and amides) (160 – 180 ppm);

carbonyl carbons (quinones, ketones and aldehydes) (180 – 220 ppm). Aliphatic alcohols and ethers (60 – 90 ppm in Fig. 4.8) were rich in NMW and HMW. However, the higher level of aromatic carbons (105 – 160 ppm) and carbonyl carbon (160 – 180 ppm) were contained in LMW. These results are consistent with the features explained from the elemental compositions and acidic functional group contents (Table 4.1).

Figure 4.9 shows the FT-IR spectra for NMW, HMW and LMW fractions. Similar spectra were obtained for NMW and HMW. The large peaks at about 1050 cm⁻¹ appeared for NMW and HMW are assigned to C-O-C stretching of ethers or C-O stretching for aliphatic alcohols [45]. The intense of these peaks suggests the higher level of ethers and aliphatic alcohols, being consistent with the higher levels of aliphatic alcohols and saccharide in the results of NMR spectra. The strong peak at around 1720 cm⁻¹ appeared for LMW is assigned to C=O stretching of carboxylic acids and/or ketones, supporting the results that the higher level of carboxylic acids is contained in the LMW. Because of differences in such structural features, the binding properties may be altered by molecular weight fractions of SWEOM.

4.3.2.2. Binding constants and capacities

From subtracting Eq. (3) from Eq. (2), the fraction of labile Fe(II)-SWEOM complexes can be written by the equilibrium as below:



The conditional binding constant of labile Fe(II)-SWEOM complexes, K_b , can be defined as:

$$K_b = \frac{[\text{Fe(II)-SWEOM}]_{\text{labile}}}{[\text{Fe(II)}]_{\text{free}}[\text{SWEOM}]} \quad (6).$$

The total concentration of binding sites for forming labile Fe(II)-SWEOM complexes

(C_L) can be expressed as:

$$C_L = \text{SWEOM} + \text{Fe(II)-SWEOM}_{\text{labile}} \quad (7).$$

The relationship between the concentrations of free Fe(II) ($[\text{Fe(II)}]_{\text{free}}$) and labile Fe(II)-SWEOM complexes can be derived by combining the Eqs. (6) and (7):

$$[\text{Fe(II)-SWEOM}]_{\text{labile}} = \frac{K_b \times C_L \times [\text{Fe(II)}]_{\text{free}}}{1 + K_b [\text{Fe(II)}]_{\text{free}}} \quad (8),$$

K_b and C_L were calculated by the non-linear least square regression analysis of the data set for $[\text{Fe(II)}]_{\text{free}}$ and $[\text{Fe(II)-SWEOM}]_{\text{labile}}$ to Eq. (8) [28,30]. The binding capacity (BC) can also be calculated as:

$$BC (\text{mol g}^{-1} \text{C}) = \frac{C_L}{[\text{SWEOM}] (\text{g L}^{-1}) \times \% \text{C}} \times 100 \quad (9)$$

where the concentration of SWEOM was 0.05 g L^{-1} , and the %C is the carbon content in Table 4.1. Figure 4.10 shows plots and fitting curves for the three fractions. The total Fe(II) concentrations varied from 0 to $28 \text{ } \mu\text{M}$. The experimental data sets were well fitted to Eq. (8) with the square of the correlation coefficients (R^2 in Table 4.3) above 0.98.

To demonstrate the validity of the above method, glutamic acid was examined as a model labile ligand, because this was one of major amino acid residues in SWEOM [18]. In this model experiment, glutamic acid and total Fe(II) concentration were 100 and 0 to $32 \text{ } \mu\text{M}$, respectively. The experimental plots were well fitted to Eq. (8) with the square of correlation coefficients ($R^2 = 0.98$). The average and standard deviations ($n = 3$) of $\log K_b$ and C_L (μM) values for glutamic acid were 5.19 ± 0.15 and 47.5 ± 9.7 , respectively. This $\log K_b$ value was in good agreement with the value from IUPAC stability constants database (5.13 at $20 \text{ } ^\circ\text{C}$ and $I = 0.65$) [46]. The C_L value indicates that glutamic acid form the dimeric complexes with Fe(II). These results indicate that the

method proposed in the present study is useful for the determination of conditional stability constant for Fe(II)-organic matter complexes under seawater conditions.

The $\log K_b$ and BC values for NMW, HMW and LMW are summarized in Table 4.3. The values for $\log K_b$ and BC for NMW and HMW were similar, consistent with the similar structural features of these fractions. Carboxylic acids and phenolic hydroxyl groups, oxygen-containing groups, are known to be major binding sites for Fe in natural organic matter, such as humic substances [14,28]. The BC value for LMW was significantly larger than those for NMW and HMW, being consistent with the trend in level of acidic functional groups in SWEOM samples (Table 4.1). Based on the HSBA principle, oxygen-containing groups are classified as hard bases [14,47], and Fe(II) is classified as a borderline acid [48]. However, nitrogen-containing groups can be classified as the borderline base that has strong affinity to Fe(II). As indicated in Table 4.3, $\log K_b$ values for HMW and NMW were somewhat larger than that for LMW. Thus, the higher $\log K_b$ values in HMW and NMW can be attributed to the contribution of nitrogen-containing groups. However, BC was governed by the dominant binding sites in the samples. The nitrogen containing groups were much smaller than oxygen-containing functional groups in all SWEOM samples (Table 4.1), indicating that oxygen-containing groups such as acidic functional groups serve as dominant binding sites for Fe(II) in SWEOM samples. These results suggest that the SWEOM, especially the LMW fraction, can contribute to supplying labile Fe(II) species from the fertilizer into seawater.

4.4. Conclusions

Labile species of Fe(II)-SWEOM complexes were determined based on the kinetics of the ligand-exchange reactions of Fe(II) complexes with SWEOM and FZ. Ascorbic acid was useful in preventing the reoxidation of Fe(II) to Fe(III) during the analysis. The method described here is effective for evaluating the lability of complex species of Fe(II) with natural organic matter under conditions analogous to seawater. The findings reported here indicate that the low molecular weight fraction of the SWEOM played an important role in supplying dissolved iron in the form of labile Fe(II)-SWEOM species.

Table 4.1. Elemental composition and acidic functional group content for the NMW, HMW and LMM fractions.

Fraction ^a	NMW	HMW	LMW
Yield (g kg ⁻¹)	1.2	1.1	1.3
Elemental composition (%)			
C	45.7 ± 0.5	43.5 ± 0.3	46.1 ± 1.3
H	6.27 ± 0.16	6.19 ± 0.02	4.74 ± 0.04
N	6.41 ± 0.12	5.83 ± 0.15	4.07 ± 0.05
O	38.1 ± 0.3	37.6 ± 0.8	38.1 ± 1.2
S	0.84 ± 0.04	0.91 ± 0.03	1.36 ± 0.02
Ash	2.74 ± 0.20	6.28 ± 0.49	6.04 ± 1.93
Atomic ratio			
H/C	1.64 ± 0.06	1.70 ± 0.01	1.22 ± 0.00
N/C	0.12 ± 0.00	0.11 ± 0.00	0.08 ± 0.00
O/C	0.63 ± 0.01	0.65 ± 0.00	0.62 ± 0.00
Acidic functional group content (mmol g ⁻¹ C)			
-COOH	3.85 ± 0.01	1.66 ± 0.17	11.5 ± 0.3
Phenolic-OH	3.75 ± 0.86	3.71 ± 0.61	8.09 ± 2.01

^a Molecular weight fractions of SWEOM: NMW > 0.5 kDa; HMW > 100 kDa; LMW < 0.5 kDa.

Table 4.2. Percentages and total amino acid residues and amino acid compositions as a result of the hydrolysis of SWEOM-NMW and LMW.

	NMW	LMW
Total amino acid contents (mg g ⁻¹ -Sample)		
	15.3	28.2
Amino acids	Compositions (%)	
Alanine (Ala)	12.3	10.2
Arginine (Arg)	3.35	3.47
Asparagine (Asn)	n.d. ^a	n.d. ^a
Aspartic acid (Asp)	11.6	15.6
Cysteine (Cys)	0.621	0.904
Glutamine (Gln)	n.d. ^a	n.d. ^a
Glutamic acid (Glu)	10.5	16.0
Glycine (Gly)	11.9	13.2
Histidine (His)	1.33	1.42
Isoleucine (Ile)	5.17	4.36
Leucine (Leu)	8.37	6.82
Lysine (Lys)	3.01	4.43
Methionine (Met)	1.08	1.61
Phenylalanine (Phe)	4.07	3.72
Proline (Pro)	4.78	5.21
Serine (Ser)	5.27	3.80
Threonine (Thr)	6.68	5.84
Tryptophan (Trp)	n.d. ^a	n.d. ^a
Tyrosine (Tyr)	2.54	2.87
Valine (Val)	7.47	0.422

^a Not detected.

Table 4.3. Conditional binding constants ($\log K_b$) for labile Fe(II)-SWEOM species and binding capacities (BC) of SWEOM to Fe(II)

Fractions ^a	$\log K_b$	BC ($\mu\text{mol g}^{-1}\text{C}$)	R^2
NMW	6.17 ± 0.09	936 ± 93	0.987
HMW	6.20 ± 0.08	992 ± 71	0.987
LMW	5.91 ± 0.12	1546 ± 215	0.993

^a Molecular weight fractions of SWEOM: NMW > 0.5 kDa; HMW > 100 kDa; LMW < 0.5 kDa.

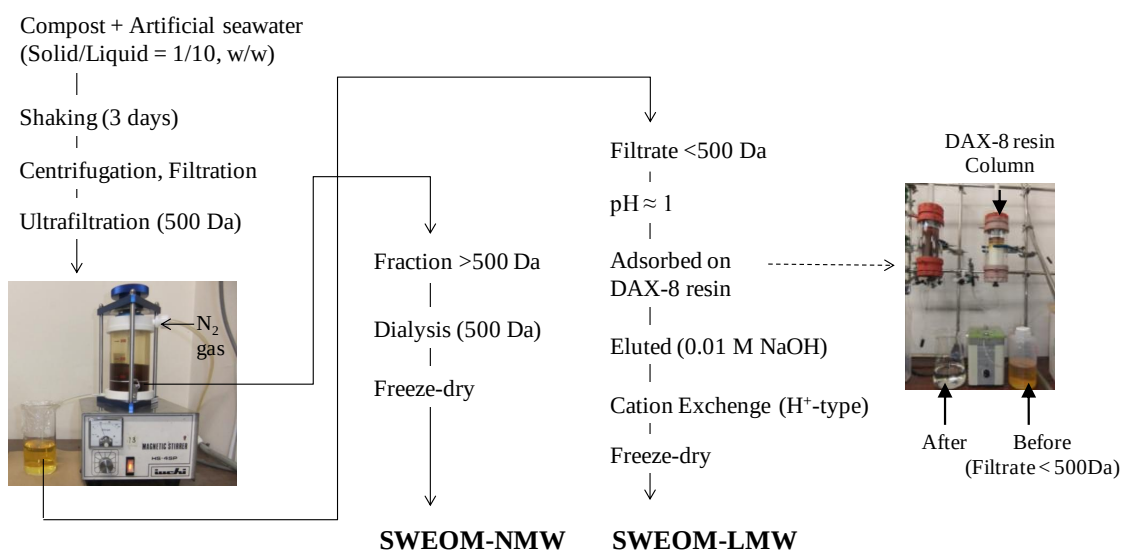


Fig. 4.1. Flowsheet of methods for extraction and molecular weight fractionation of SWEOMs.

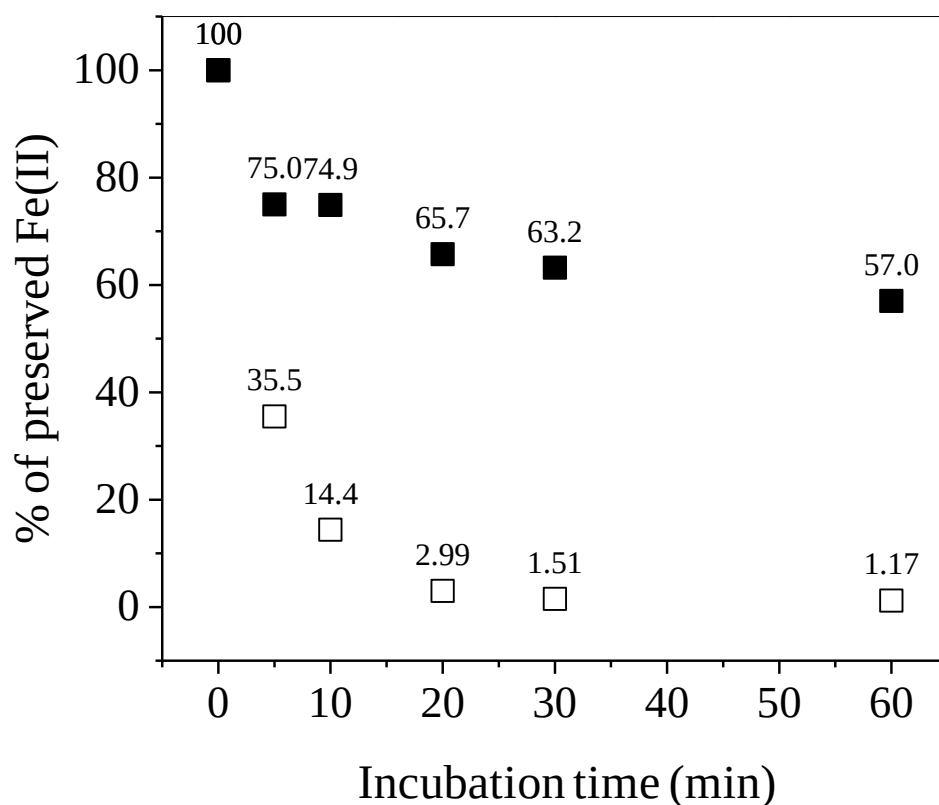


Fig. 4.2. Kinetics for the bleaching of color for Fe(II)-FZ complex. Closed and open squares denote reactions run in the presence (20 mM) and absence of ascorbic acid, respectively. Total injected Fe^{2+} concentration was 50 μM . Average values are labeled on each plot (S.D. = 0 – 2.1, $n = 3$).

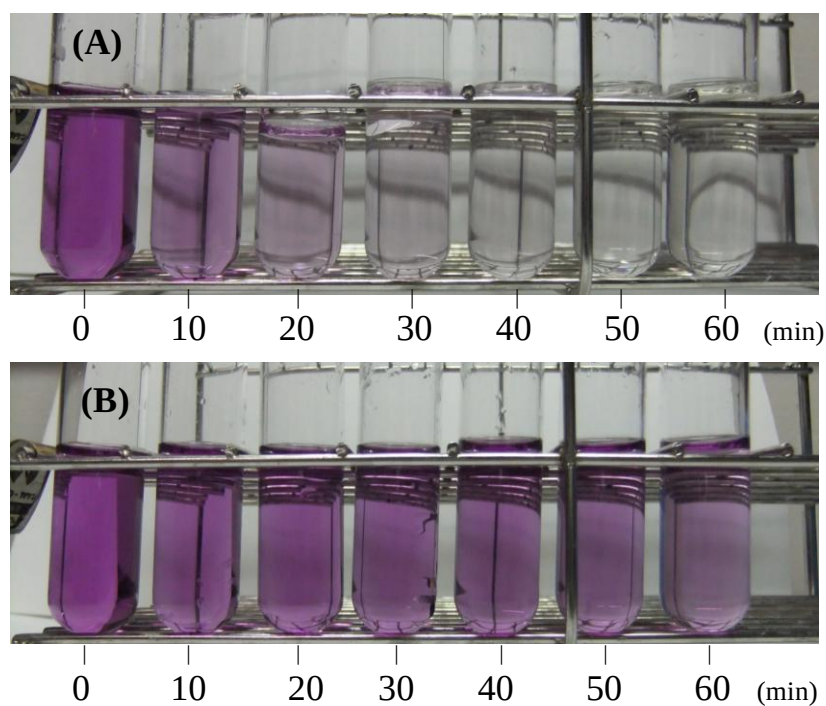


Fig. 4.3. Coloration of Fe(II)-FZ complexes under artificial seawater buffer (pH 8) in the absence (A) or presence (B) of ascorbic acid. Labeled values represent interval times (min) between addition of Fe(II) and FZ. The pictures were taken after 10 min from addition of FZ.

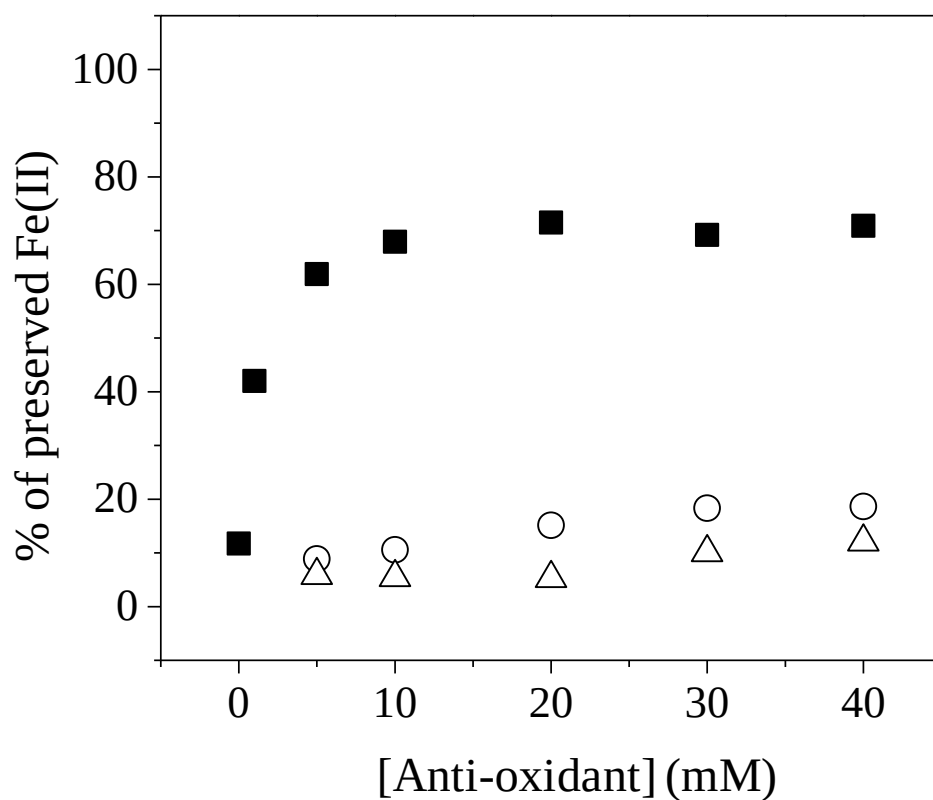


Fig.4.4. Effect of anti-oxidant concentration on preserving Fe(II). Symbols: ■, ○ and △ represent ascorbic acid, hydroxylamine and glucose, respectively (S.D. = 0.1 – 4.4, $n = 3$). Total Fe^{2+} concentration was 50 μM . Incubation time from point of ferrozine addition was 30 minutes.

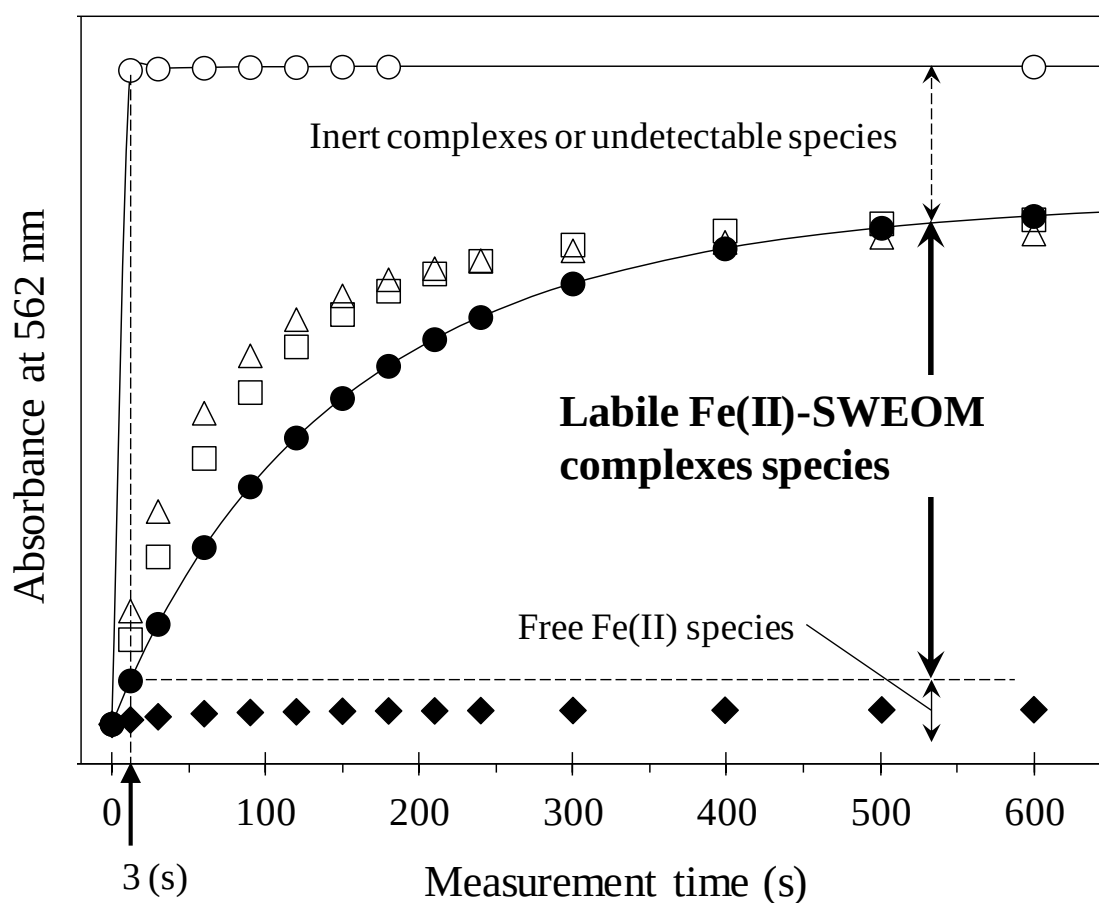


Fig. 4.5. Kinetics of the increase in absorbance at 562 nm as the result of forming Fe(II)-FZ complexes in the absence and the presence of SWEOM, and a schematic diagram for determining the free species of Fe(II) and labile Fe(II)-SWEOM complex species. Added Fe concentration was 10 μM . Symbols: ○ represents the complexation reaction between ferrozine and Fe^{2+} ; ●, □ and △ represent the ligand-exchange reactions between ferrozine and the labile Fe(II)-SWEOM complex in the presence of SWEOM 50, 25 and 10 mg L^{-1} ; ◆ represents the kinetic curve for the solution in the presence of SWEOM alone (50 mg L^{-1}).

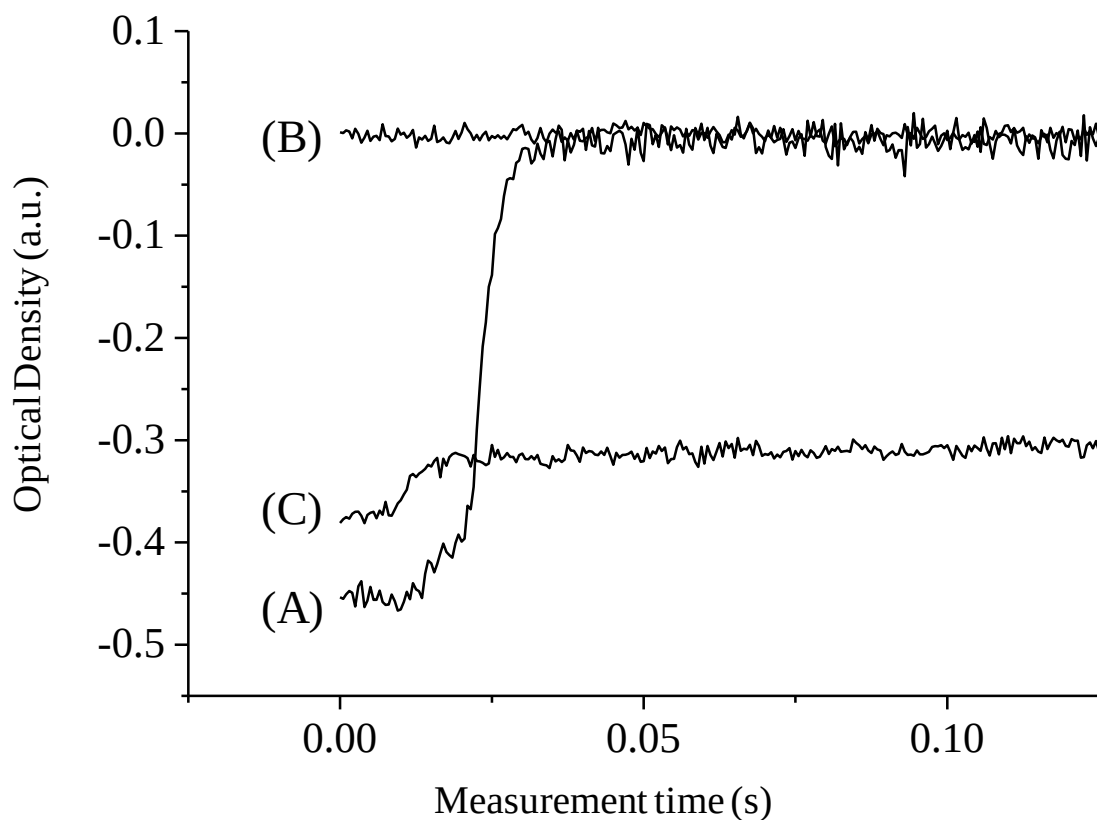


Fig. 4.6. The kinetics curves for the formation of Fe(II)-FZ complex by the stopped-flow spectrometry. Kinetic curves were collected under the seawater conditions: pH 8, $I = 0.7$, [Ascorbic acid] = 10 mM; (A) FZ + Fe²⁺, (B) SWEOM + FZ, (C) Fe(II)-SWEOM + FZ. The initial [Fe(II)] for (A) and (C) were prepared to 30 μM . SWEOM (LMW) for (B) and (C) were prepared to 50 mg L^{-1} .

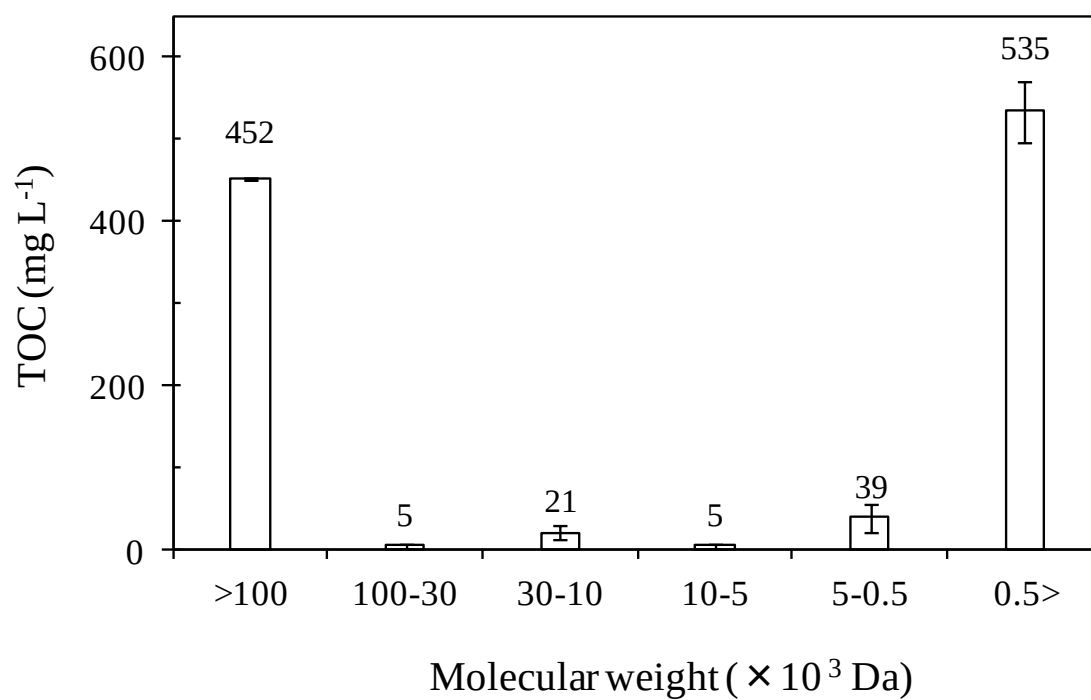


Fig. 4.7. Distribution of molecular weight fractions in the SWEOM.

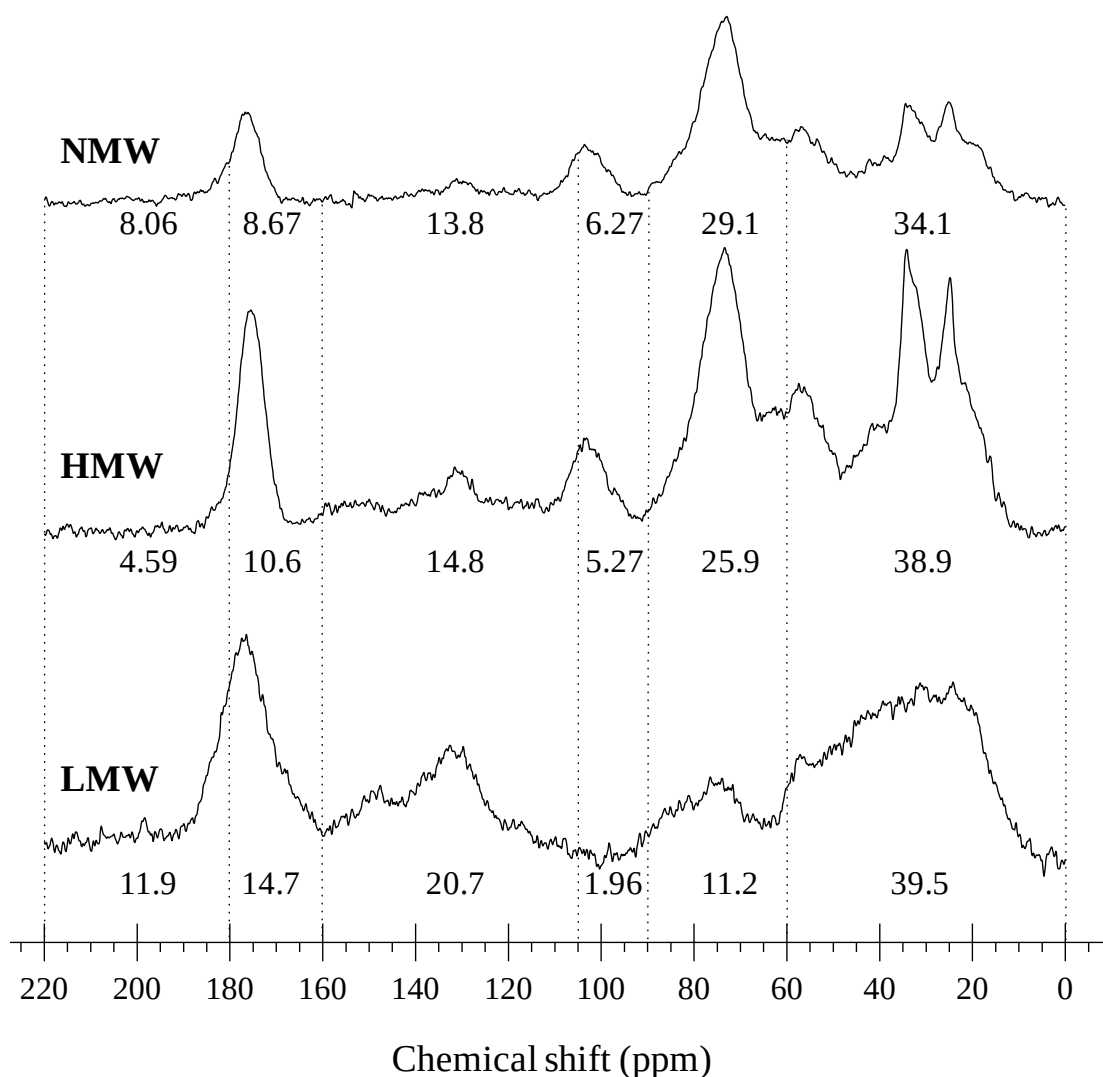


Fig. 4.8. Solid-state CP-MAS ^{13}C NMR spectra for SWEOM-NMW, HMW and LMW fractions. The labeled values indicate the compositions (%) of carbon species for each area corresponding to each chemical shift ranges. 0 – 60 ppm: acrylic carbons. 60 – 90 ppm: aliphatic alcohols and ethers carbons. 90 – 105 ppm: anomeric carbons. 105 – 160 ppm: aromatic carbons. 160 – 180 ppm: carbonyl carbons (acids, esters and amides). 180 – 220 ppm: carbonyl carbons (quinines, ketones and aldehydes).

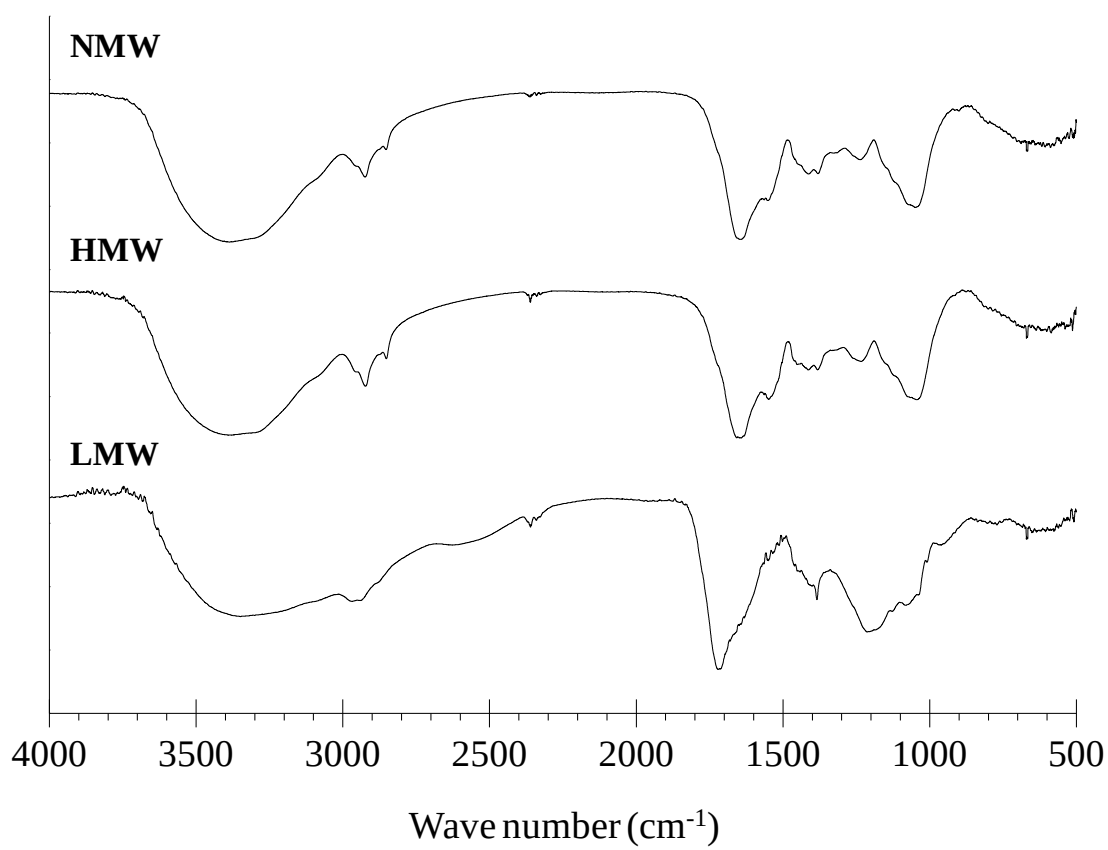


Fig. 4.9. FT-IR spectra for SWEOM-NMW, HMW and LMW fractions.

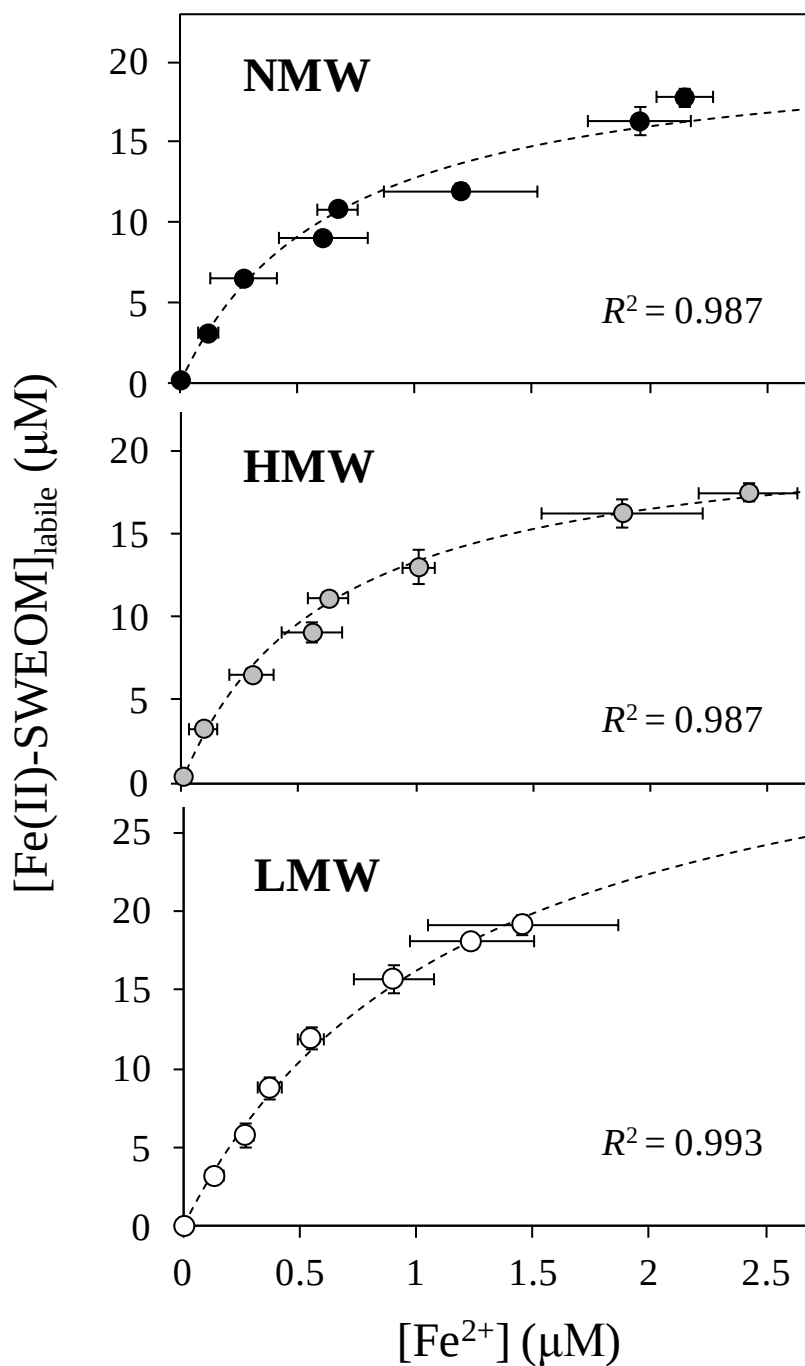


Fig. 4.10. Relationships between Fe(II) and labile Fe(II)-SWEOM concentrations at $[\text{SWEOM}] 50 \text{ mg L}^{-1}$: NMW (a), HMW (b) and LMW (c). Error bars represent standard deviations ($n = 4 - 5$).

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Chapter 5

***Bioavailability of iron complexed with
seawater extractable organic matter: Effect on
the gametogenesis for brown macro algae
(Laminaria Japonica)***

5.1. Introduction

Seaweed beds have important roles in the protection of ecosystems, supplying useful materials for our society and the fixation of carbon dioxide [1,2]. Recently, seaweed depletion has become to be the serious problem in coastal areas all over the world, especially in Japan coastal areas [3-9]. As described in previous chapters, iron is an essential nutrient for growth of marine algae and an important factor for algal biomass [1,10,11]. As explained in section 1.2 (Fig. 1.3 in Chapter 1), uptake of the dissolved iron is required for the reproductive growth (gametogenesis) of the gametophyte in the case of in brown macroalgae [12-15]. Thus, the lack of bioavailable iron in seawaters of coastal areas can lead to the caused depletion of sporophytes, that is, to barren ground [14-17].

It has been known that natural organic matter plays important roles in mobility, water solubility and bioavailability of trace metal ions in aquatic environments. Indeed, the majority of dissolved iron in seawater is present as complex species with dissolved organic matter (DOM) [18]. In addition, the Fe-EDTA complex is reported to be more bioavailable form than amorphous iron like hydrous oxides and hydroxides [14,15]. In the fertilization technique to restore the seaweed-bed [19], SWEOM from the compost can serve as a chelator of iron to elute bioavailable species from steel slag. Although SWEOM can form the complex with Fe(II) under the conditions of seawaters (pH 8, $I = 0.7$) [20-22], it has not been known whether the Fe-SWEOM complex is bioavailable species for brown macro algae or not. Therefore, the effects of the Fe-SWEOM complex on the growth of brown macro algae should be elucidated. Although bioavailability of Fe to microalgae (phytoplankton and diatoms) has been investigated by many

researchers [10,23-31], there are a little information for brown macro algae (e.g., seaweed) [16,17].

In the preliminary test, the formation to sporophyte was observed by culturing of male and female gametophyte in the presence of Fe and SWEOM as a chelator (Fig. 5.1). In the chelate free medium (Fig. 5.1, A and B), the maturation and sporophyte formation were rare, even in the presence of the higher Fe concentration (10 μ M, B in Fig. 5.1). In addition, significant effects on maturation and sporophyte formation were also observed in the presence of SWEOM, even in the lower concentration of Fe (1 μ M) in which the sporophyte was not formed in chelate free medium. Protandrous or simultaneous reproductive growth was found on algal bisexual gametophytes [32]. Moreover, the sporophytes are formed by the cell differentiation of the matured and fertilized female gametes [1]. Thereby, the maturation of female gametophyte is a key process in the lifecycle of brown algae, and this is relating to the restoration of seaweed-bed. In this chapter, the effect of SWEOM on the bioavailability of Fe was examined by the maturation (i.e., gametogenesis) of the female gametophyte for brown macro algae (*Laminaria Japonica*). First, the effects of bioavailable Fe species on the gametophyte, the influence of iron and chelate concentrations on the gametogenesis were examines by using Fe-EDTA complexes.

5.2. Materials and Methods

5.2.1. Materials

(+)-Biotin, thiamin hydrochloride, sodium metasilicate nonahydrate, 1,2-dichloroethane and 1-chlorobutane were purchased from Wako Pure Chemical

Industries, Ltd. (Osaka, Japan). *Beta*-glycerophosphate disodium salt hydrate and ferrichrome (iron-free form *Ustilago sphaerogena*) were obtained from Sigma (St. Luis, USA). *Ortho*-fluorotoluene and vitamin B₁₂ were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). *N,N'*-di(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid (HBED) monohydrochloride was purchased from Sterm Chemicals, Inc. (Massachusetts, USA). Tris(hydroxymethyl)aminomethane (Tris), sodium bromide, boric acid, strontium chloride hexahydrate and cobalt (II) chloride hexahydrate were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Nitrilotriacetic acid (NTA), potassium iodide, disodium molybdate (VI) dehydrate, lithium chloride and manganese (II) chloride tetrahydrate were obtained from Junsei Chemical Co., Ltd. (Tokyo, Japan). Zinc chloride and Rubidium chloride were purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan) and Kishida Chemical Co., Ltd. (Osaka, Japan), respectively. The above chemicals were obtained as special reagent grade and used without further purification. The compost sample, which prepared by maturing the mixture of bark-tips and cowpat, was obtained from Mori Industry Co., Ltd (Hokkaido, Japan). The compost samples was freeze-dried and passed through a 2 mm mesh stainless steel sieve. Compost particles less than 2 mm were used in the all experiment. Other chemicals were obtained as special reagent grade from Wako Pure Industry (Osaka, Japan)

5.2.2. Preparation of SWEOM

SWEOMs were extracted, purified and fractionated, as described in section 2.2.4 and 4.3.2.1 [20-22]. SWEOM was extracted from a bark compost with artificial seawater (pH 8.1) by shaking for 3 days under darkness and N₂ atmosphere. The

suspension was centrifuged, and the supernatant was filtered through an ADVANTEC 5A filter paper. The filtrate was fractionated by ultrafiltration membrane for > 500 Da (NMW) and < 500 Da (LMW). After the fractionation, NMW was purified using a dialysis tube (molecular weight cut-off of 500 Da) against ultrapure water. The LMW was acidified (pH 1 ~ 2) and adsorbed on DAX-8 resin. After rinsing with ultrapure water, the adsorbed LMW was eluted with 0.01 M NaOH aqueous. The eluent was passed through a H⁺-type cation exchange resin column (DOWEX[®] HCR-W2). These purified SWEOM fractions were obtained as powdered samples by freeze-dry. To avoid denaturation, SWEOM powders were stored in bio-freezer (– 30 °C). Stock solutions of SWEOMs (500 mg L⁻¹) were prepared by dissolving powdered samples in 0.05 M NaOH aqueous.

5.2.3. Culturing gametophytes of brown macro algae

The gametophyte of *Laminaria japonica* was employed for experiments in this chapter. The stock was obtained from Professor Taizo Motomura in Muroran Marine Station, Field Science Center for Northern Biosphere, Hokkaido University. The gametophyte was precultured from zoospores, which were collected from the matured sporophytes of *Laminaria japonica* at Charatsunai (Murooran, Hokkaido, Japan) and washed with a capillary pipette method. The gametophyte was sterilely preserved in a 30 mL grass test tube that includes 10 – 15 mL of ASP₁₂ NTA (Fe-free) medium [12,13] at 10°C under the conditions of 14h:10h light and dark cycle from a dim light (ca. 10 μE m⁻² s⁻¹) with fluorescence light. Compositions of ASP₁₂ NTA [33] are summarized in Table 5.1. Before using, all medium and pipettes using for all culturing and inoculation were sterilized by autoclave (121 °C, 20 min). In order to maintain the sterilized

conditions, all tubes or flasks using in culturing were maintained sealing with Aluminum-foil except for working in clean bench.

5.2.4. Gametogenesis test

Figure 5.2 shows a flowsheet of the preparation of medium and culture of gametophyte. Erlenmeyer grass flasks used for experiments were soaked in 0.1 M HCl aqueous for 1 day and rinsed with pure water. Subsequently, these flasks were covered with Al foil, heat to sterilized (235 °C, 3 h) and stored until using. Stock solutions of Tris (1.0g / 10 mL, pH 7.8), Na₂SiO₄·9H₂O (150 mg/10 mL), β-glycerophosphate disodium salt(10 mg/1 mL), NaNO₃ (100 mg/1 mL), K₃PO₄ (10 mg/1 mL), vitamin B₁₂ (0.2 mg/1 mL), biotin (0.1 mg/1 mL), thiamin (10 mg/1 mL), ZnCl (1.04 mg/1 mL), MnCl₂·4H₂O (14.40 mg/1 mL), CoCl₂·6H₂O (0.4 mg/1 mL), NaBr (128.62 mg/5 mL), SrCl₂·6H₂O (60.77 mg/1 mL), RbCl (2.83 mg/1 mL), LiCl (12.21 mg/1 mL), Na₂MoO₄·sH₂O (12.61 mg/ 1 mL) and KI (0.13 mg/100 μL) were prepared by dissolving each reagent in ultra pure water. Vitamin mix (vitamin B₁₂ 0.2 μg, biotin 1.0 μg and thiamin 100 μg are contained in 1 mL of water). The PII and SII metals were prepared from above stock solutions (Table 5.1), and used for the preparation of ASP₁₂ medium. These stock solutions were stored in cool (5 °C) and darkness after adding a volatile preservative (*o*-fluorotoluene : 1,2-dichloroethane : 1-chlorobutane = 1 : 1 : 3 (volume basis)). This volatile preservative can be eliminated, when the sample is autoclaved. The stock solution of Fe²⁺ (1 or 10 mM) was prepared by dissolving Fe(SO₄)₂(NH₄)₂·6H₂O in 0.01 M HCl aqueous. The 90 % volume of ASP₁₂ medium was prepared as described in Table 5.1, while NTA, Na₂EDTA and FeCl₃ were eliminated when using for gametogenesis tests. NTA is employed to enhance the chelating

capability in the medium. EDTA is especially needed for dissolving Fe in the ASP₁₂ medium. Therefore, the trace metal ions including in SII and PII metals can be dissolved in the ASP₁₂ medium (Fe, EDTA-free). The chelating agents or SWEOM were added to the 90 % volume of the ASP₁₂ medium (Fe, EDTA-free), the pH of the mixture was adjusted to 7.8 – 8.0 with dilute HCl or NaOH aqueous, and this was then diluted to the fixed volume with pure water. A 30 mL aliquot of the medium was pipetted into 100-mL Erlenmeyer grass flasks. Subsequently, A 0 – 30 μ L aliquot of 1 or 10 mM Fe²⁺ stock solution was added to these media. After sealing with Al-foil, these media were sterilized using autoclave (121 °C, 20 min). It was confirmed that the TOC values and UV-vis absorption spectra of SWEOM in the medium were not changed before and after autoclaving. Thus, SWEOM in the media are not significantly degenerated or mineralized by autoclaving. The gametophyte was inoculated into the cooled media under HEPA condition. In order to avoid contamination of chelating agents (i.e., EDTA and NTA), the gametophyte was washed with the sterilized ASP₁₂ medium (Fe, EDTA-free) under HEPA condition before the inoculation. Prior to inoculation, arbitrary volume of sterilized ASP₁₂ medium (Fe, EDTA-free) was placed to the test tube, and the gametophyte was then cut into small peace with a Pasteur pipette under HEPA condition (Fig. 5.2). After the inoculation, the gametophyte was cultured for 14 days at 10 °C under the conditions of 14h:10h light and dark cycle of 20 – 40 μ E m⁻² s⁻¹ with LED light.

5.3. Results and Discussions

5.3.1. Evaluation of the maturity

The female gametophyte can alter to oogonium when cultured in the suitable condition. The photomicrographs of cell-morphosis, which is found in the reproductive growth of the female gametophytes for *Laminaria japonica*, is shown in Fig. 5.3. When the gametophytes (Fig. 5.3 (A)) were grown reproductively, their cells were transformed to gametes and released oogonium from the inside of the cell wall (spherical cells in Fig. 5.3 (B)). An enlarged photomicrograph and detailed explanation of matured gametophyte cell were shown in Fig. 5.3 (C) (the detailed photomicrographs of the egg releasing process had been reported by Lüning et al [34]). Therefore, the matured gametophyte can be clearly distinguished by microscopic observation of their cell form (Fig. 5.3). At arbitrary incubation period, population of the gametophyte in the medium was evaluated by observing an inverted microscope. The degree of maturation was estimated as a percent of maturity [12,13], according to the following equation:

$$\text{Maturity(\%)} = \frac{\text{Population of matured gametophyte}}{\text{Population of total gametophyte}} \times 100$$

where “matured” and “total gametophyte” represent the population of gametophyte that is induced oogonium and the sum of population for the matured and unmatured gametophytes (counted up to 100 ~ 200 of gametophytes), respectively.

5.3.2. Influences of environmental factor, Fe and EDTA concentrations on gametogenesis

Some environmental factors, which can affect the egg release in gametophyte of brown macro algae, had been investigated. Lüning and Neushul [34,35] showed the influence of light and temperature on the reproductive growth. In their report, various *Laminaria* gametophytes living in California were saturated for vegetative growth with $20 \mu\text{E m}^{-2} \text{ s}^{-1}$ of irradiance, while more irradiance ($40 - 60 \mu\text{E m}^{-2} \text{ s}^{-1}$) was required for

the reproductive growth [1,35]. For temperature, the reproductive growth was saturated at 12 °C, while this was inhibited in lower (7 °C) and higher (17 °C) temperature, and no gametophytes were reproductive grown at 20 °C [35]. Lüning [1,34] showed that blue light was required for the gametogenesis. In addition, the gametophyte that is irradiated by red or green light results in only vegetative growth, and the eggs (oogonia) were released in night of the experimental daily cycle (light and dark cycles). Motomura et al. [12,13] investigated the effect of nutrients on the gametogenesis of laminarial gametophyte using the ASP₁₂ NTA medium, showing that higher concentration of iron above 9 µM is required for the gametogenesis, while the oogenesis is inhibited in the presence of the higher concentration of boron above 92 µM with 1.78 µM of Fe. Suzuki et al. [14] reported that the oogonia remarkably formed in the lower levels of iron (0.4 – 2 µM of Fe(III)-EDTA in seawater passed through a 0.45-µm filter), compared to the case of Motomura et al. (ca. 10 µM) [12,13]. The reproductive growth on algae is dependent on their environmental conditions, because a reproductive growth takes with strongly heteromorphic generations (i.e., growth in different from the conformity to environmental changes) and it is a key to the specific survival [1]. Thus, in order to understand the influences of Fe and chelate concentrations in detail, the gametogenesis was examined using the modified ASP₁₂ medium under the conditions for suitable light irradiation and temperature.

Figure 5.4 shows that kinetics of the female gametophyte (*Laminaria japonica*) in the presence (■: 1 µM) and absence (□) of Fe with 1 µM of EDTA. In the presence of Fe, the oogonia were appeared and increased after the 7 – 14 day period, as described in other reports [12-14]. Although the Fe concentration was lower than that in other reports [12-14], the effect of Fe on the reproductive growth was confirmed and the maturity

was reached to approximately 80 % after a 14-day period. The maturity did not increase significantly after 14 days, while parthenogenetical formed sporophytes appeared. Therefore, maturity after a 14-day period was employed in the present study.

Figure 5.5 shows the maturities after a 14 day period cultured in the modified ASP₁₂ media that contained a variety of Fe concentrations in the presence (■, 1:1 molar ratio to Fe) and absence (□) of EDTA. In spite of the increased [Fe], the majority of gametophytes were not transformed to oogonia in the absence of chelator (□ in Fig. 5.5), and only 8 % of gametophyte was observed 10 μM of [Fe]. Fe(OH)₃ is a major species of Fe in the chelate free medium [36]. Therefore, the result in Fig 5.5 □ indicates that Fe(OH)₃ is not available species for the maturation of gametophyte. These results are consistent with the results by Suzuki et al. [14] that the approximately 30 % of maturity is observed after a 15-day period at 2 μM of Fe in the medium of EDTA-free, which is prepared by passing seawater through a 0.45-μm filter. This can be attributed to the fact that DOM such as siderophore may serve as chelator of iron. Crystalline ferric oxides, such as goethite (γ-FeOOH) and hematite (Fe₂O₃), cannot be source of bioavailable Fe for algae, while colloidal Fe(OH)₃ can be available for vegetative growth on phytoplankton [36]. However, Fe(OH)₃ is not available for the reproductive growth, because of the lower solubility and slower dissolution [2,14]. On the other hand, in the presence of EDTA (1:1) media (■ in Fig 5.5), the maturities dramatically increased with an increase in Fe concentration of 0.1 – 1 μM. However, maturity reached plateau above 1 μM of Fe with 1 μM of EDTA. These results suggest that Fe-EDTA complex can be bioavailable species to gametophyte, and the 1 μM of Fe is sufficient concentration level for the oogenesis.

Figure 5.6 shows the percents maturity after a 14-day period in a variety of EDTA

concentrations (■: [Fe] 1 μ M, □: Fe-free). When the EDTA concentration increased to 0.1, 0.5 and 1 μ M in the presence of 1 μ M of Fe (■ in Fig. 5.6), the percent maturity drastically increased to 9, 64 and 84 %, respectively. This can be due to an increase of Fe-EDTA complex, which can be regarded as a bioavailable species (Fig. 5.4 and 5.5). When the EDTA concentration increased to 10, 30, 50 and 100 μ M at 1 μ M of Fe, the percents maturity decreased to 64, 17, 4 and 0 %, respectively. After 30 days, no increase of the maturities was observed, and the gametophyte continued vegetative growth (Fig. 5.7 (B,C)). However, for the molar ratio of Fe:EDTA = 1:10, the maturity increased to approximately 90 %, and some oogonia were transferred to sporophytes by parthenogenesis (Fig. 5.7 (A)) [37]. In the absence of Fe condition, only the gametophyte with vegetative growth was observed, even in the higher EDTA concentration. These results show that EDTA has no adverse effect on the vegetative growth in the applied concentration ($\sim$ 100 μ M). However, excess of free EDTA would inhibit the Fe uptake of the gametophyte in the stage of oogonia formation.

5.3.3. Hypothesis of Fe uptake mechanism

Mechanisms for metal uptake into algae have mostly been investigated for microalgae (phytoplankton) [24,27-29,30,38-43]. Based on the concepts and data results in previous reports, three types of mechanisms for the Fe-uptake into the gametophyte have been proposed, as illustrated in Fig. 5.8. Cell membrane for algae is covered with cell wall, which is constructed with fiber matrix: the reticulum matrix components of brown algal wall are composed of cellulose microfibrils, alginate network, xylo-fuco-glucans or glyconans, homofucans and glycoproteic linkages [1]. Thus, the route of ion uptake into cells is from cell surface. Subsequently, iron passes through the

cell wall and plasma membrane (lipid bilayer) to reach to the cytoplasm [1]. In addition, this cell wall can serve as a filter (~ 10 nm of pore diameter [44]), in which, the larger molecules like DOM, may not be passed though. Thus, small molecule like Fe-EDTA complex can pass though the cell wall. After accessing to the plasma membrane, the Fe-EDTA (Fe-L) complex can be taken as follows: (1) uptake directly (Fig 5.8 (1)), (2) uptake by binding the dissociated Fe with binding site (X) on the membrane (Fig. 5.8 (2)), (3) uptake *via* the formation of ternary complex with X (X-Fe-L) (Fig. 5.8 (3)).

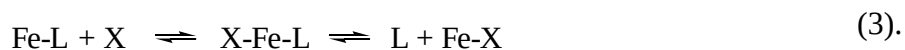
The Fe-EDTA complex cannot be taken up directly, because Fe uptake remarkably decreased in the presence of excess EDTA. In general, the Fe uptake mechanism (1) in Fig 5.8 has been employed in microorganisms and/or terrestrial plants. They can directly uptake complex species of Fe with siderophore, which are low-molecular-weight organic ligands secreted when lacking Fe. However, Böttger et al. [45] elucidated the Fe uptake strategy of *Ectocarpus siliculosus*, which is a model brown alga, from the genomic interpretation. They suggested that *E. siliculosus* does not use a siderophore, and employs ferrireductase and Fe(II) transport protein [45]. In *Phaeodactylum tricornutum* for a model diatom, the ferrireductase activity of the cells precultured under the Fe-limited condition was enhanced [46]. Therefore, the mechanism (1) in Fig 5.7 is unlikely for the Fe uptake by the gametophyte. If the gametophyte would employ the ferrireductase as a binding site for the Fe uptake (mechanism 2), Fe uptake could be written as follows:



where L and X represent organic ligand, such as EDTA, and binding site on the cell

membrane, respectively. As suggested by many researchers (e.g. [28]), free Fe is bioavailable species and this can yield *via* dissociation reaction of the complex, as shown in Eq. (2). When existing excess L, dissociation equilibrium can shift to left side, and the concentration of bioavailable Fe may decrease [28], being consistent with the result in Fig. 5.6.

However, Aristilde et al. [38] reported that marine phytoplanktons can take up Cu complexes (Cu-L) by their binding site on the cell membrane *via* formation of the ternary complexes (X-Cu-L), suggesting the presence of a strong binding site to Fe, X, in a gametophyte cell membrane. Formation of the ternary complex between strong chelate (OP) and Fe(II)-SWEOM was described as a ligand-exchange reaction in Chapter 3 [21]. If the Fe-SWEOM complex (Fe-L) would be taken uptake *via* formation of ternary complex (X-Fe-L), the reaction could be written as following equilibrium:



In the presence of excess L, the equilibrium (3) would shift to left side and lead to the decrease of Fe-X level *via* the dissociation. The excess L can inhibit the ligand-exchange reaction between L and X. This reaction is plausible, based on the result in Fig 5.6. The sum of Eqs. (1) and (2) can derive the simple ligand-exchange reaction as below:



Sutak et al. [47] mentioned that, for diatoms, binding of iron to cell surface is a key critical step before the uptake, and the reaction can thermodynamically be controlled. In contrast, Motomura et al. [12,13] reported that the gametogenesis of *L. japonica* did not occur in the presence of 1 μM Fe as an EDTA complex. Such the difference can be

explained, based on Eq. (4). Motomura et al. [12,13] used the media, which was prepared by adding Fe-EDTA complex (1:1 molar ratio) to the ASP₁₂ NTA medium (Fe-free). The default ASP₁₂ NTA media (Fe-free) contains 28 μM of EDTA and 523 μM of NTA. As shown in Fig. 5.6, the present maturity was decreased in the presence of excess EDTA for Fe:EDTA = 1:30. Thus, the equilibrium of Eq. (4) could not shift to right side by the excess free ligands when using the ASP₁₂ NTA medium.

5.3.4. Effect of SWEOM on the gametogenesis

Effects of SWEOM on supplying bioavailable Fe to gametophyte were examined in the modified ASP₁₂ (EDTA-free) medium, which contained various SWEOM concentrations; 0, 1, 5, 10, 20 and 50 mg L^{-1} . The results are shown in Fig 5.9. In the SWEOM-alone condition, 4 ~ 30 % of gametophytes were matured. Such the maturations may be induced by trace Fe remained in SWEOM. The NMW and LMW samples contained 18 and 8 $\mu\text{mol-Fe g}^{-1}$, respectively (see in Chapter 4 and [22]). In the presence of NMW (5 mg L^{-1}) and Fe added (1 μM), the maturity was reached to 50 %, while the percent maturities were not changed in 10 and 20 mg L^{-1} of NMW (■ in Fig. 5.9 (A)). This indicates that Fe-NMW complexes can reach to cell membrane and pass through the cell, because average molecular weight of NMW was approximately 30 kDa (Table 2.2 in Chapter 2, [20]). However, in the higher concentration of SWEOM in the addition of 1 μM Fe, NMW was obviously precipitated as colloids materials (Fig. 5.10). The flocculation is enhanced in the presence of Fe [18]. Thus, aggregation of NMW leads to the precipitation of Fe and the decrease of bioavailable Fe species. In contrast, the maturities were increased with an increasing in LMW concentration (■ in Fig. 5.9 (B)). The 25 ~ 60 % of maturity was observed in the lower concentration of LMW (1 ~

5 mg L⁻¹), and it was then reached to 70 % for 10 mg L⁻¹ and 75 % for 20 mg L⁻¹ of LMW. The colloidal materials were not observed, even in the higher concentration of LMW (Fig. 5.10). Humic substances and natural organic matter like the SWEOM sample does not contain only the complex species of Fe *via* coordination but also the loosely bound Fe *via* electrostatic interactions [18]. To elucidate the influence of Fe species with binding loosely, the gametogenesis was tested using polyacrylic acid (PA of 5000 Da for a common size of aquatic DOM [18]) as a model polyelectrolyte. Only 2 ~ 3 % of gametophytes were matured in the presence of 1 or 10 mg L⁻¹ PA, although loosely bound Fe with DOM could serve as bioavailable species. Thus, loosely bound Fe *via* electrostatic interaction cannot serve as bioavailable species.

To examine relation of the complexation affinity with gametogenesis, concentrations of Fe-SWEOM complex species were calculated using logK_b and binding capacity [22] by the IUPAC SC-Database [48] by consideration competitive complexation with Ca²⁺ (10 mM) and Mg²⁺ (50 mM) that were major concomitant divalent cations in the seawater (Table 5.2). SWEOM samples included trace iron as impurities. Initially contained Fe in LMW (column of “initial” in Table 5.2.) was regarded as inert complex species because no gametogenesis was observed in the presence of LMW alone. In contrast, initially contained Fe in NMW was considered as labile complex species, because 4 ~ 30 % maturation was occurred in the presence of NMW alone. Although the Fe-L concentrations for LMW were estimated to be lower than those for NMW (Table 5.2), the obtained percent maturity for LMW is higher than those for NMW (■ in Fig. 5.9). The decrease of percent maturity in the higher concentration of LMW (50 mg L⁻¹) may be due to the presence of excess ligand (Fe:L = 1:35), which can inhibit ligand-exchange reaction to yield labile species of Fe. These

results indicate that LMW is more useful fraction to serving bioavailable Fe than NMW.

5.3.5. Comparison of percent maturity in a variety of chelators

Effect of various chelators on the maturity of gametophyte was investigated. Fig. 5.11 shows maturities after a 14-day period of cultivation in the modified ASP₁₂ medium, which contained various chelators and Fe (1 μ M). As shown in Fig. 5.11, chelating agents were needed for the maturation of gametophyte. In particular, EDTA was the highest effective chelator of all tested. HBED is known to be stronger than that of EDTA and form inert complex with iron. Catechol (Cat) and protocatechuic acid (PCA) are used as model chelators of siderophore that contains the catechol type binding sites [1,30]. Ferrichrome (FC) is a siderophore containing the hydroxamate type binding sites [50]. In the presence of 1 μ M HBED, FC, Cat and PCA, the percent maturity remarkably decreased to below 10 %. In particular, no gametophyte maturation was observed in the case of FC and HBED, suggesting that Fe may not be taken into the gametophyte. The logK values for Fe-L referred from IUPAC stability constant database are summarized in Table 5.2 [48]. The ligand-exchange reaction to X (Eq. (4)) may hardly occur in the presence of strong chelator ($\log K > \text{Fe-EDTA}$). Similar reason can be considered in the cases of 1:2 complexes for Cat and PCA, $\text{Fe}(\text{Cat})_2$ ($\beta_2 \approx 35$) and $\text{Fe}(\text{PCA})_2$ ($\beta_2 \approx 33$). It can be considered that a few percents of maturity observed at 1 μ M of Cat and PCA were due to the minor distribution of 1:1 complexes, Fe-Cat ($\beta_1 = 20$) and Fe-PCA ($\beta_1 = 19$). Thus all Fe species form 1:2 complexes (FeL_2 type) in the presence of 10 μ M of Cat and PCA, and bioavailable iron may not be supplied to the gametophyte, being consistent with the results in other report [30].

Figure 5.12 shows relationship between [Fe-L] and percent maturity observed

after 14-day period in the presence of Fe (1 μ M). In the presence of EDTA (0 ~ 1 μ M), the [Fe-L] clearly correlated to percent maturity ($R^2 = 0.972$). The linear line for this relationship may serve the threshold for the bioavailable concentration of Fe-L. From the plot in Fig. 5.12, Fe-NTA ($\square$ in Fig. 5.12) can be estimated as bioavailable species such as Fe-EDTA, although FC and HBED can be regarded as unbioavailable Fe-L species ($\star$ in Fig. 5.12). In the case of SWEOM, except for initially contained Fe in LMW (opened squares of red color in Fig. 5.12), the majority of points were in the vicinity of the correlative line. Thus, plots for Fe-LMW complex in the upper left region of the correlative line, indicate the bioavailable species of Fe-LMW. These results lead to a conclusion that SWEOMs, especially LMW, can serve as effective chelating agent to supply dissolved iron for maturing gametophyte.

5.4. Conclusion

The results in this chapter suggest that the gametophyte takes bioavailable Fe *via* the ligand-exchange reaction between DOM and binding site on the cell membrane of algae. Thus, bioavailability of Fe is dependent on the concentrations of chelating agents and/or conditional binding constants of the iron complexes. SWEOM fractions from compost can serve as a chelator to supply the bioavailable species of Fe to gametophyte (*L. japonica*). Although NMW was flocculated due to the aggregation *via* the complexation of Fe, LMW was anticipated to an effective SWEOM fraction for supplying highly bioavailable species of Fe in seawater.

Table 5.1. Compositions in 1000 mL of the ASP₁₂ NTA medium (pH 7.8 – 8.0) [33].

ASP ₁₂ NTA (1000 mL, pH 7.8 - 8.0)			
NaCl	28.0 g	Na ₂ SiO ₃ ·9H ₂ O	150 mg
KCl	0.7 g	SII metals [†]	10 mL
MgSO ₄ ·7H ₂ O	7.0 g	PII metals [‡]	10 mL
MgCl ₂ ·6H ₂ O	4.0 g	Vitamin B ₁₂	0.2 µg
CaCl ₂	1.11 g	Biotin	1 µg
NaNO ₃	100 mg	Thiamine	100 µg
K ₃ PO ₄	10 mg	NTA [*]	100 mg
Na ₂ -glycerophosphate	10 mg	Tris	1.0 g
[‡] PII metals (100 mL)		[†] SII metals (100 mL)	
Na ₂ EDTA [*]	100 mg	NaBr	128.62 mg
FeCl ₃ [*]	2.98 mg	SrCl ₂ ·6H ₂ O	60.77 mg
H ₃ BO ₃	114.33 mg	RbCl	2.83 mg
MnCl ₂ ·4H ₂ O	14.40 mg	LiCl	12.21 mg
ZnCl ₂	1.04 mg	Na ₂ MoO ₄ ·2H ₂ O	12.61 mg
CoCl ₂ ·6H ₂ O	0.4 mg	KI	0.13 mg

^{*} Ligands and Fe in the default compositions were eliminated in the ASP₁₂ used for the gametogenesis experiment in the present study.

Table 5.2. Calculated concentrations of Fe complexes species in the presence of various ligands with considering the competitive complexation with Ca^{2+} and Mg^{2+} .

Chelator	logK			mg L^{-1}	[L] μM	[FeL] ([FeL ₂)] μM	
	FeL (FeL ₂)	CaL (CaL ₂)	MgL (MgL ₂)			initial [†]	final [‡]
NMW	6.20*	3.10 [§]	1.60 [§]	1	0.43*	0.02	0.03
				5	2.14*	0.09	0.14
				10	4.28*	0.18	0.27
				20	8.56*	0.36	0.51
LMW	5.90*	3.10 [§]	1.60 [§]	1	0.70*	0.01	0.02
				5	3.49*	0.04	0.08
				10	6.97*	0.08	0.16
				20	13.9*	0.16	0.29
				50	34.9*	0.40	0.62
EDTA	25.3	11.0	9.20		1.00		0.76
NTA	16.0	6.53	5.50		1.00		0.50
Cat	20.1 (34.7)	1.70	2.00		1.00		0.07 (0.48)
					10.0		0.00 (1.00)
PCA	19.0 (33.3)	3.71 (6.36)	5.67 (9.84)		1.00		0.06 (0.46)
					10.0		0.00 (1.00)
FC	28.5	unkown	unkown		1.00		1.00
HBED	39.2	9.39	10.61		1.00		1.00

Stability constants were referred from previous reports: * [22], [§] [49].

[†]Initially contained Fe in SWEOM as an impurity were calculated from detected value in a previous report [22]. This Fe was considered as complexed species.

[‡][Fe-L] values after Fe adding (1 μM) were simulated using the IUPAC SC-Database.

Ca^{2+} (10 mM) and Mg^{2+} (50 mM) were used for the calculation as competing cation.

$\log\beta_1$ and β_2 of Fe-OH were also taken into consideration to 11.0 and 21.7, respectively.

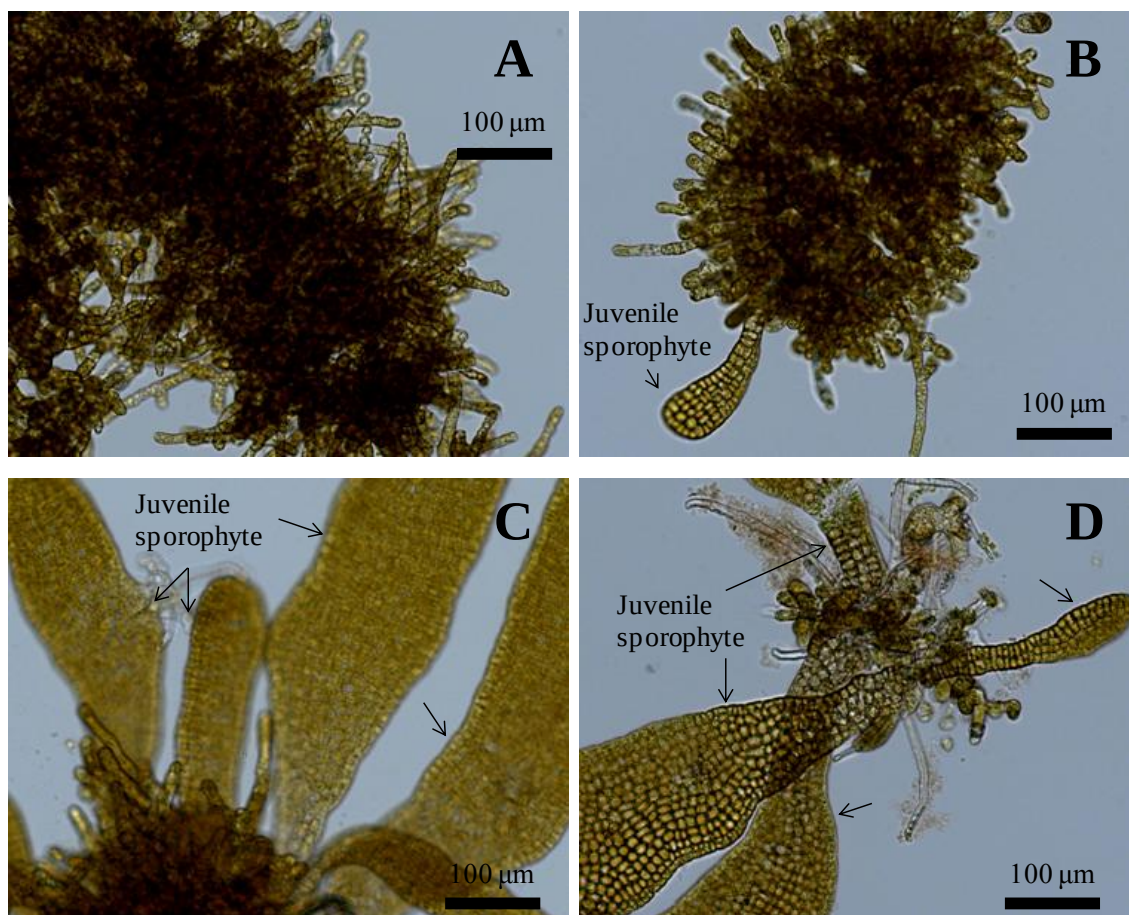


Fig. 5.1. Photomicrographs of the cultured gametophyte (male and female) in ASP₁₂ medium for 1 month under suitable condition. A and B show female gametophytes cultured in chelate-free medium: [Fe] 2 μ M (A), 10 μ M (B). C and D show female gametophytes cultured in 5 mg L⁻¹ of SWEOM: [Fe] 1 μ M (C), 5 μ M (D). Fe and EDTA in default compositions of the ASP₁₂ were eliminated in all media. Matured and fertilized female gametes formed to juvenile sporophytes (except for A).

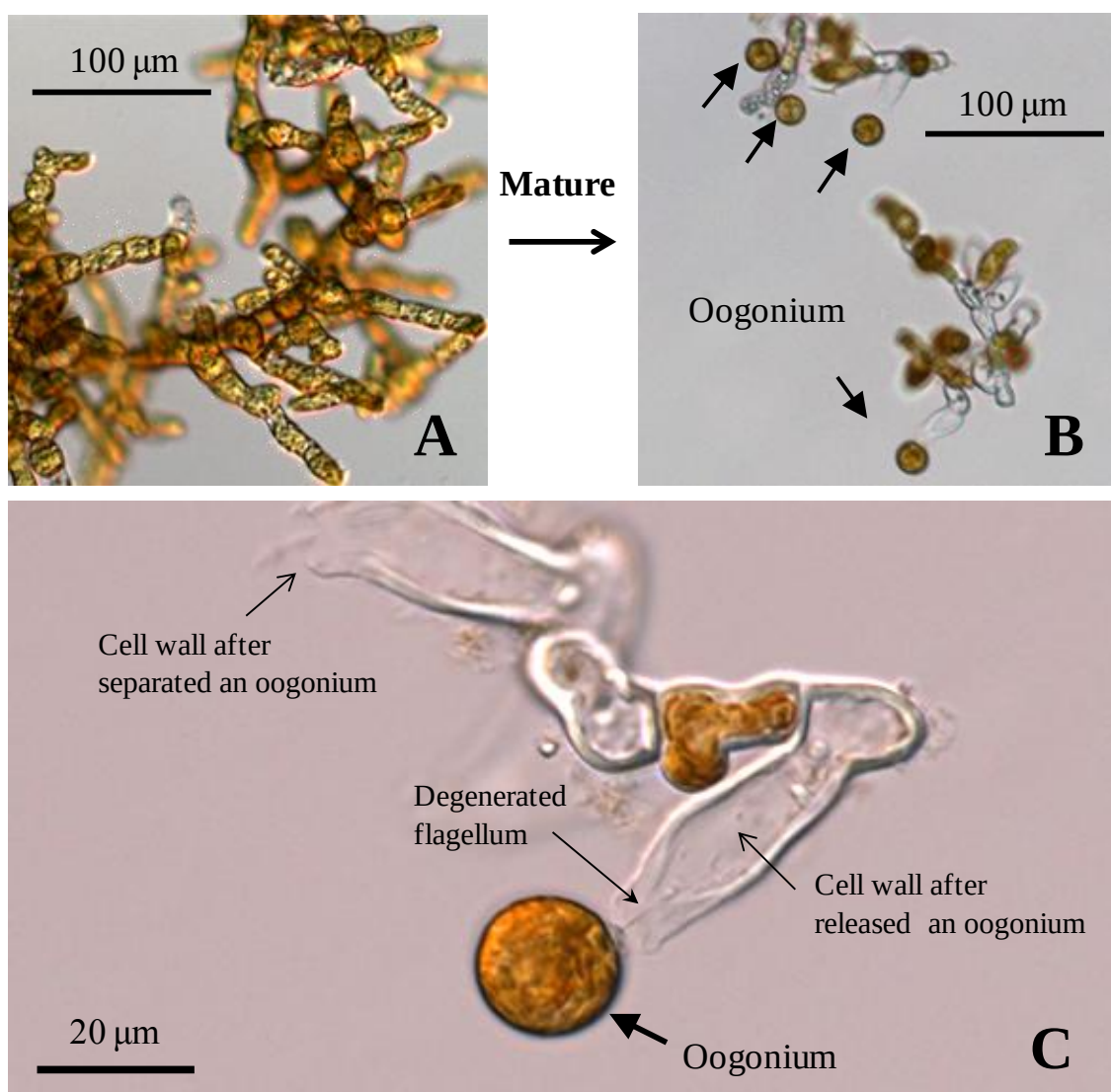


Fig. 5.3. Photomicrographs of the female gametophyte (*Laminaria japonica*) of before (A) and after maturation (B). An enlarged photograph of the oogonium (C) indicates more detailed comments of the cell.

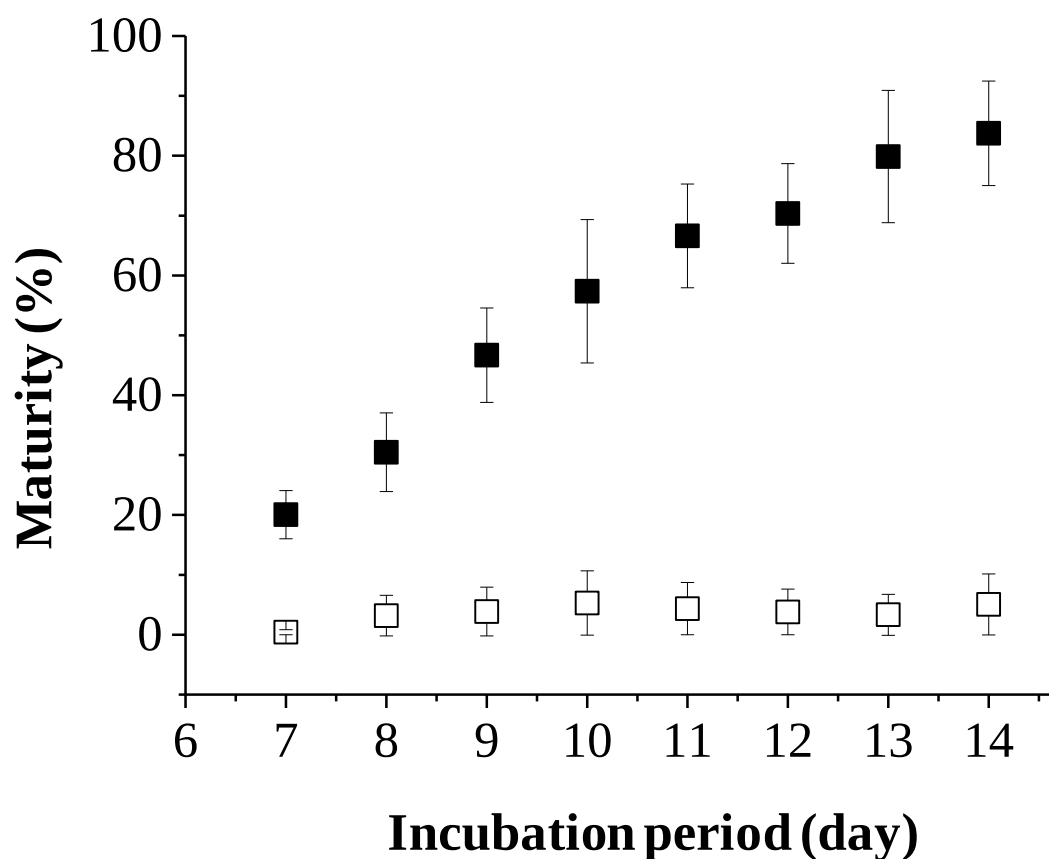


Fig. 5.4. Relationship between percent maturity (%) and incubation period (day). Filled and open squares represent the percent maturity in the presence of Fe (1 μM) as complexes with EDTA (Fe:EDTA = 1:1 ($\mu\text{M}/\mu\text{M}$)) and absence of Fe (EDTA 1 μM), respectively. Plot and Error bar represents average value and standard deviation, respectively (n = 4).

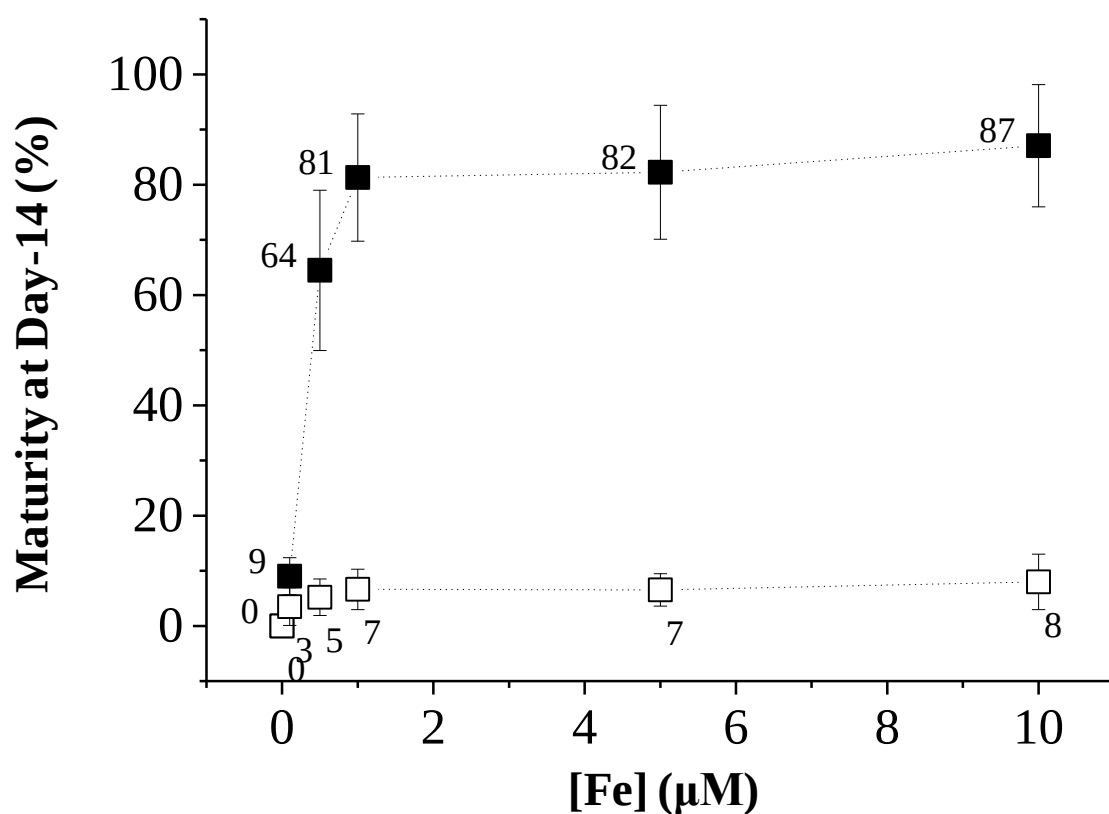


Fig. 5.5. Effect of Fe concentration on the percent maturity (%) after a 14-day period. Filled and open squares represent in the presence (Fe:EDTA = 1:1) and absence of EDTA. Plots and labeled values on the side of plots represent average maturity (%). Error bar represents the standard deviations ($n = 4$).

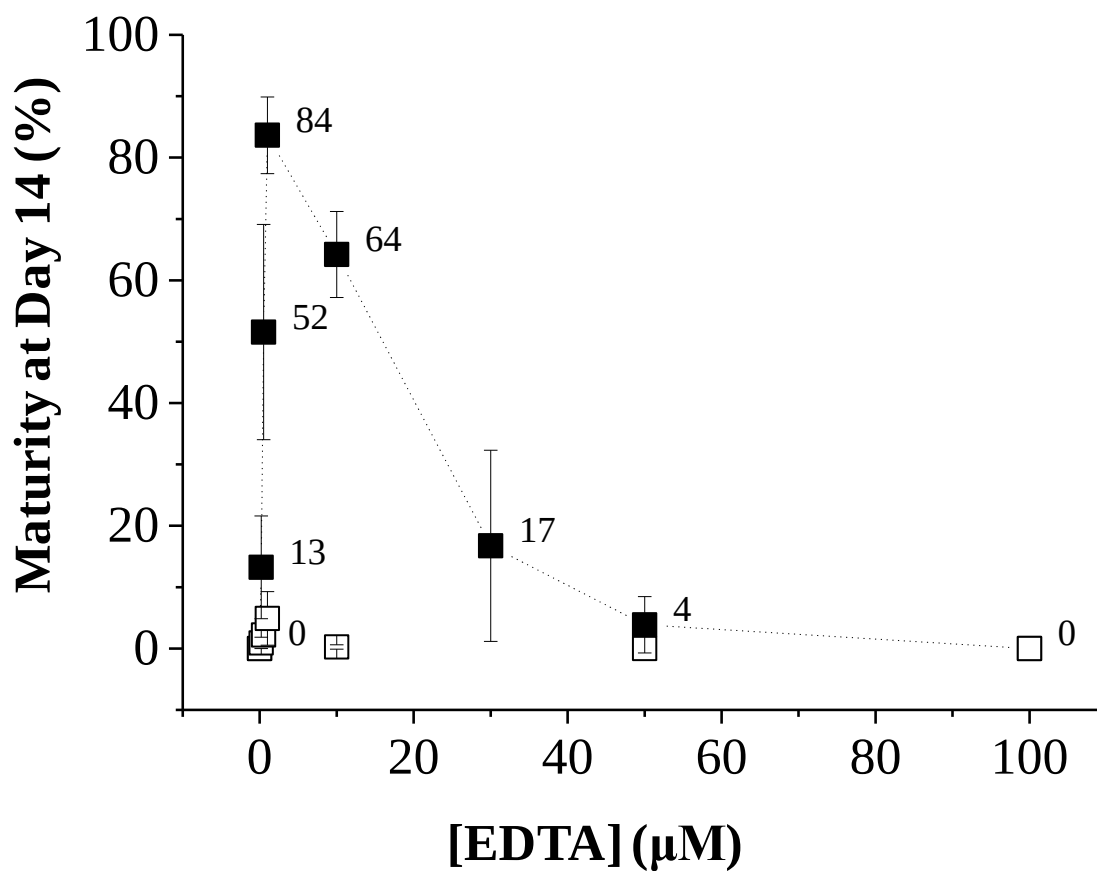


Fig. 5.6. Effect of EDTA concentration on the percent maturity (%) for a 14-day period. Filled and open squares represent the percent maturity in the presence (1 μM) and absence of Fe, respectively. Labeled values are average maturities of the each filled plots. Plots and error bars represent the average values and standard deviations ($n = 4$).

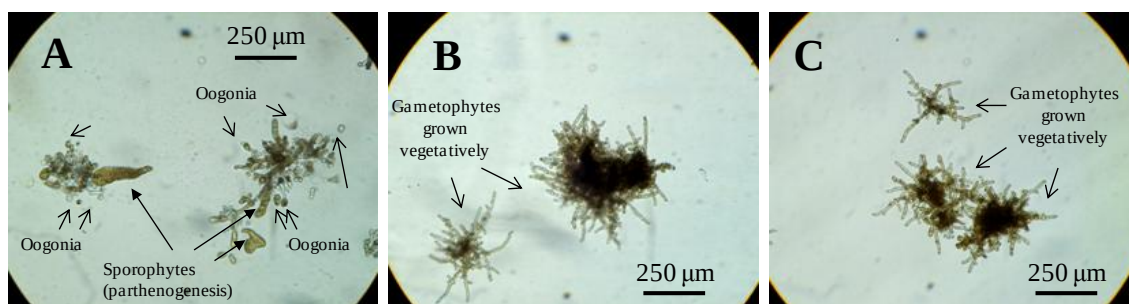


Fig. 5.7. Photomicrographs of the female gametophytes (*Laminaria japonica*) cultured for 30 days in the modified ASP₁₂ medium. Fe and EDTA concentration: A 1 and 10 μM, B 1 and 50 μM, C 1 and 100 μM. A: many oogonia were induced (ca. 90 % of maturity) and parthenogenetic sporophytes were grown. B and C: oogonia were not induced. However gametophytes grew vegetatively into branched uniseriate filamentous.

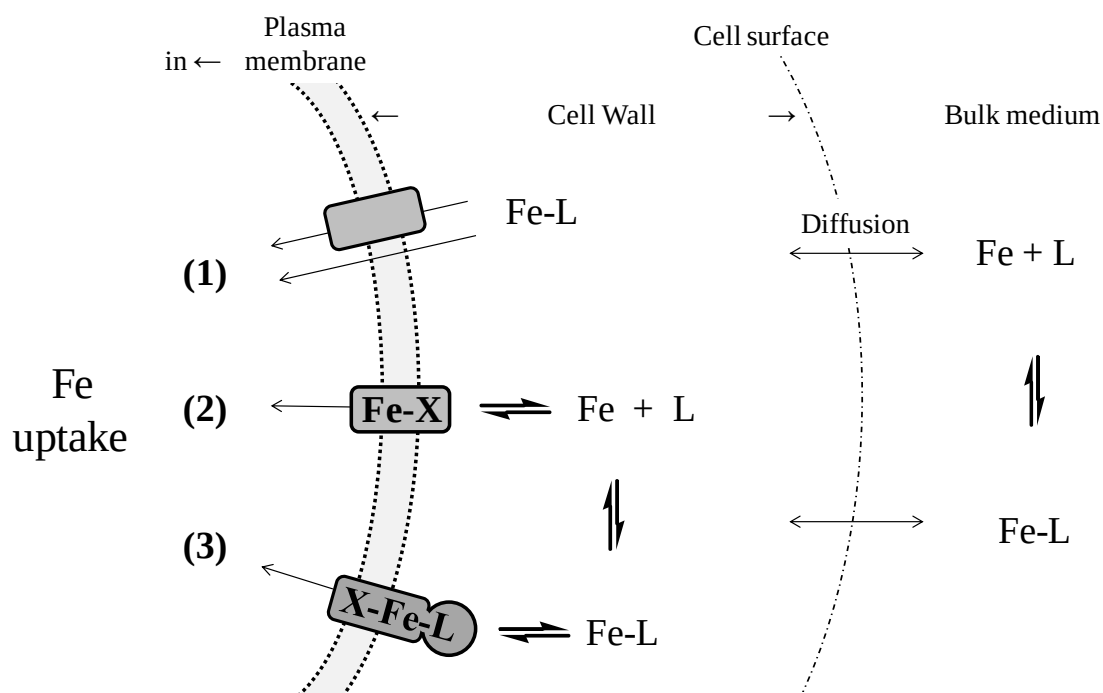


Fig. 5.8. Hypothesized mechanisms for Fe-uptake in the gametophyte of brown macroalgae. L and X represent organic ligand and binding site on the cell membrane, respectively.

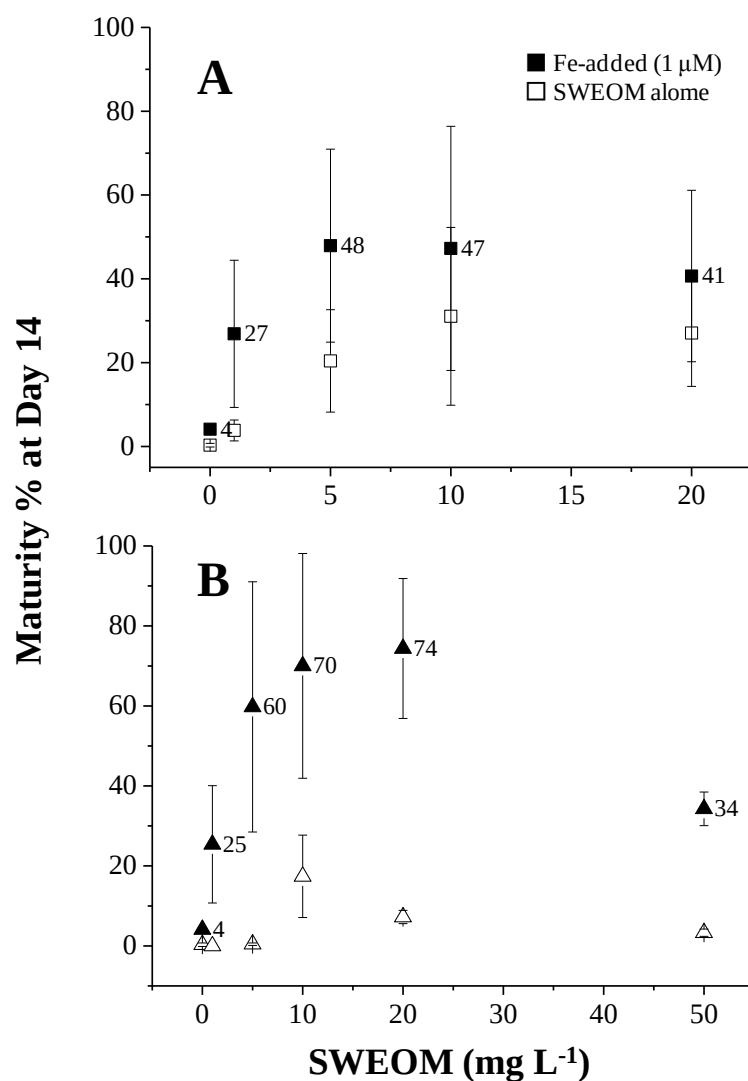


Fig. 5.9. Relationship between concentrations of SWEOMs (A: NMW, B: LMW) and percent maturity (%) for a 14-day incubation period. Filled, open plots and error bars represent the average maturity under Fe added (1 μ M), SWEOM alone, and standard deviation ($n = 4$), respectively. The percents maturities in the addition of Fe are labeled on the side of filled plots.

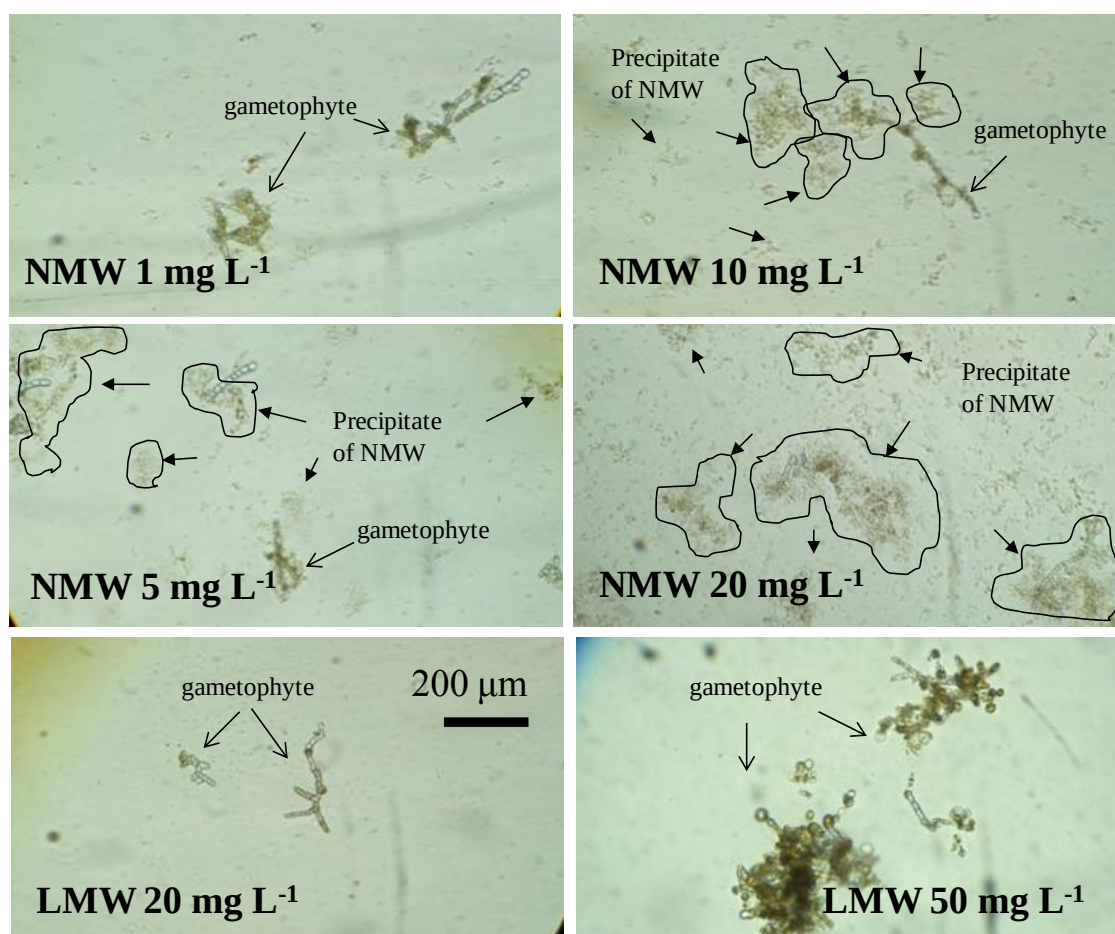


Fig. 5.10. Photomicrographs for the precipitation of SWEOM colloids in the presence of Fe (1 μM). These photomicrographs were taken after a 14 day period of the cultivation. The NMW and LMW concentrations were shown at the lower left of each photomicrogram.

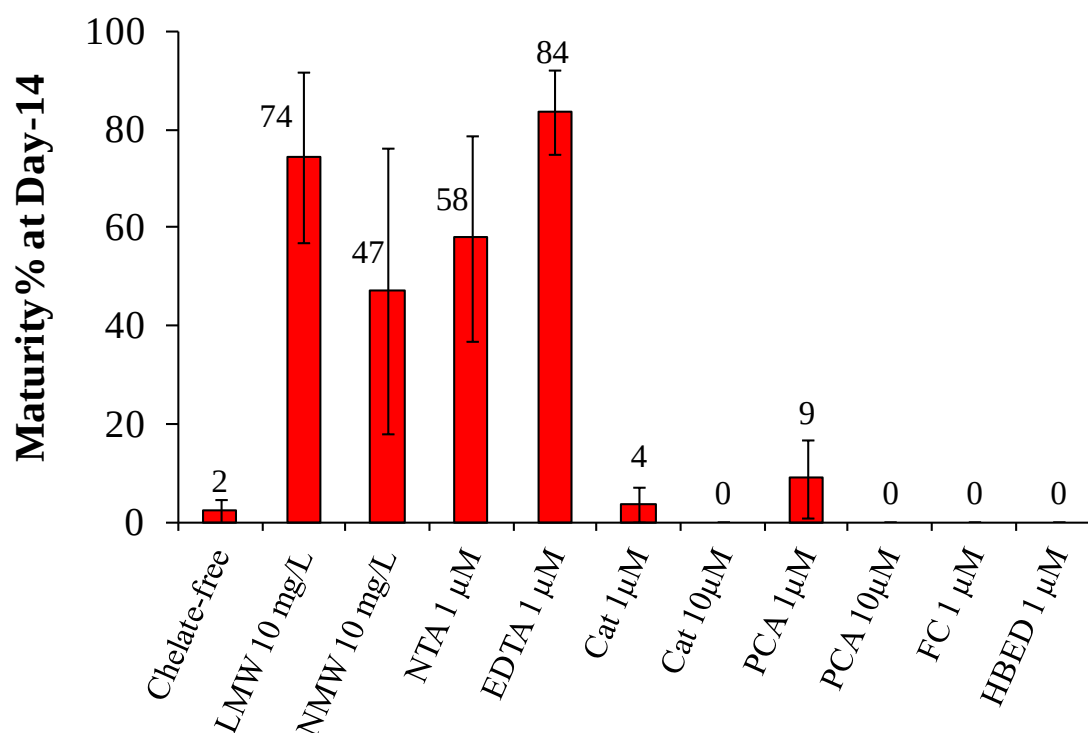


Fig. 5.11. Percent maturity for a 14-day cultivation of female gametophyte (*L. japonica*) in the modified ASP₁₂ (Fe, EDTA-eliminated) media adding various chelating agents and Fe 1 (μ M). Cat, PCA and FC represent catechol, protocatechuic acid and ferrichrome, respectively. Values labeled on top of the bar and error bar represent average values (%) and standard deviations, respectively ($n = 3 \sim 5$). In the presence of various chelating agents (except for SWEOM), percent maturity in the absence of Fe were in the range of 0 ~ 2 %.

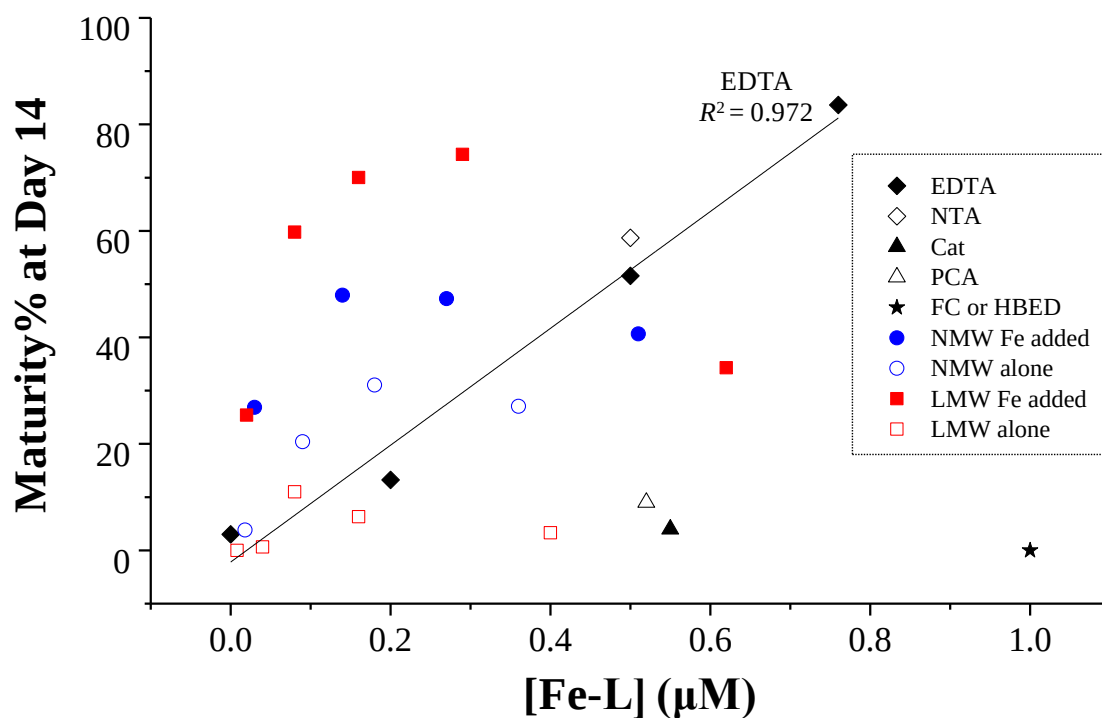


Fig. 5.12. Relationship between [Fe-L] and Maturity% at 14 incubation days in Fe adding condition (1 μM). [Fe-L] represents concentration of complexed Fe species calculated by IUPAC SC-Database, in the presence of Ca^{2+} (10 mM) and Mg^{2+} (50 mM) as competing cation in the ASP_{12} medium. Concentrations of EDTA and other model chelators were 0 ~ 1 μM and 1 μM , respectively.

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Chapter 6

Summary

Seaweed beds do not play important roles in only the protection of coastal ecosystems but also in carbon fixation. Thus, seaweed depletion significantly affects fisheries, coastal ecosystems and global warming. Recently, deforestation of seaweeds due to sub-urban development of coastal areas, destruction of seaweed habitat and some environmental changes is known as a serious environmental problem in coastal areas all over the world. Extinction of seaweed species was first recorded at Sydney in 2003. In Japan, the barren ground of coastal areas due to a variety of environmental factors is also recognized as a serious coastal problem, which has been called “*Isoyake*” after the 1800s. Among various factors, Fe-lacking is considered as one of reasons for barren ground. For example, although Fe concentration was below 2 nM in seawater of barren ground, several-ten nM level of Fe were detected in seawater of the reforested coastal area. It has been known that Fe is an essential nutrient on the life stage of reproductive growth for brown macro algae, which is a preliminary life stage to form sporophyte for *Laminarian* gametophyte. If the barren ground could be defined as depletion of sporophytes, the Fe-lacking would be a plausible reason. Because dissolving Fe^{2+} and Fe^{3+} is very difficult in oxic seawaters (pH 8, $E_h = 0 - 0.3$ V). Under such conditions, soluble form of Fe is complex species with DOM. HA and FA as typical fractions of HS among DOM have binding capabilities to metal ions, and have been known to play important role in speciation, solubility and bioavailability of metal ions in aquatic environments. Based on this, a restoration technique has been examined by supplying dissolved Fe using a fertilizer comprised of steel slag and compost containing abundant HSs. However, it has not been elucidated what types of DOM from the compost are effective in binding with Fe and in availability of Fe-DOM complexes to the growth of seaweed. Because HA and FA are coagulated in the higher ionic strength like seawater,

the DOM fractions extracted with seawater from the compost can govern the speciation of dissolved Fe. In the present study, fractionation and purification methods for seawater extractable organic matter (SWEOM) as a novel DOM fraction were established, and structural characteristics of SWEOM were compared with HA and FA fractions, which were derived from the same compost origin. In addition, dissociation kinetics of Fe(II)-SWEOM complexes were investigated in the acetate buffer at pH 3.6. In this experiment, the $\Delta H^\ddagger$ for Fe(II)-SWEOM was significantly lower than those for Fe(II)-HA and FA complexes, suggesting that the Fe(II)-SWEOM complex may be more bioavailable species than the Fe(II)-HS complexes. However, determination of complexing abilities of SWEOM to Fe^{2+} under seawater conditions is required for evaluating the availability of Fe species to algal growth. However, it is difficult to determine the Fe(II)-SWEOM complex in oxic coastal seawater, because free Fe(II) species can easily be oxidized to Fe(III) and immediately form insoluble hydroxides. In the present study, a novel artificial seawater buffer was designed using a reducing agent, which cannot affect the complexation equilibrium between Fe(II) and SWEOM. In addition, the labile Fe(II)-SWEOM complex and free Fe(II) were determined by the ligand-exchange reaction with FZ and this was applied to the evaluation of Fe(II)-binding abilities of SWEOM. Finally, the effect of Fe-SWEOM complexes on the reproductive growth of gametophyte for *Laminaria japonica* was investigated. In conclusion, SWEOM can play an important role in supplying dissolved Fe as bioavailable form to seaweeds in the barren coastal areas. Outlines in each chapter are summarized as below:

In chapter 1, reasons for barren ground were explained and the required investigations were extracted, based on the previous reports. First, Fe deficiency is one

of reasons for barren ground, and a restoration technique has been examined, based on this. In addition, role of Fe for the growth of brown macro algae was explained, based on their life history. In particular, the life stage for the reproductive growth of gametophyte is one of critical process in the reforestation. Furthermore, DOM governs the speciation and bioavailability of trace metal ions in aquatic environments. Thus, significance in evaluating complexing abilities of DOM to Fe under seawater conditions was explained.

In chapter 2, extraction and purification methods for SWEOM, which is considered as a DOM fraction from the fertilizer, were developed and structural features of SWEOM were compared with those of HA and FA. H/C, N/C atomic ratios and amino acid contents of SWEOM were much higher than those for HA and FA, indicating that SWEOM contains the higher level of unsaturated hydrocarbons and proteins. The O/C ratio for SWEOM was similar to that for FA, although acidic functional group contents in SWEOM were smaller than those in HA and FA, suggesting that the larger levels of oxygen-containing functional group such as ethers and alcohols were contained in the SWEOM. In addition, SWEOM included the higher levels of fatty acids and sterols, compared to HA and FA. These results indicate that the SWEOM is comprised of lipids and proteins derived from microorganisms, and can be regarded as a novel fraction of DOM.

In chapter 3, dissociation kinetics of Fe(II)-SWEOM in acetate buffer at pH 3.6 were investigated, based on the kinetic measurement of ligand-exchange reaction with OP. Activation parameters, such as activation energy ($\Delta G^\ddagger$), enthalpy ($\Delta H^\ddagger$) and entropy ($\Delta S^\ddagger$), for Fe(II)-SWEOM were evaluated, based on the Eyring plot using pseudo-first-order rate constants of dissociation reaction at arbitrary temperatures, and

these values were compared with those for HA and FA. The $\Delta H^\ddagger$ for Fe(II)-SWEOM complex was significantly smaller than the corresponding values for Fe(II)-HS complexes. Therefore, Fe(II)-SWEOM complex is more dissociable than the Fe-HS complexes, suggesting the bioavailability of Fe(II)-SWEOM complex.

In chapter 4, speciation analysis of Fe(II)-SWEOM complex and free Fe^{2+} was investigated under seawater conditions (pH 8, $I = 0.7$). The FZ can form the stable complex with free Fe(II) of purpled color. The labile Fe(II)-SWEOM species can be quantitatively determined, based on a ligand-exchange reaction between FZ and SWEOM. However, the analysis was difficult because Fe(II) was immediately oxidized to Fe(III) to form $\text{Fe}(\text{OH})_3$ under the seawater conditions (pH 8, $I = 0.7$). To suppress the oxidation of Fe(II), the addition of antioxidants to the buffered seawater was examined. It was found that ascorbic acid was the most effective antioxidant in the preservation of Fe(II) species. Conditional stability constants ($\log K_b = 5.8 - 6.3$) and binding capacities ($900 - 1500 \mu\text{mol g}^{-1}\text{C}$) of labile complex species of Fe(II)-SWEOM were evaluated, proving that SWEOM can serve as chelator of Fe under seawater condition.

In chapter 5, the bioavailability of Fe-SWEOM was investigated by evaluating maturity of female gametophyte (*Laminaria japonica*), which was obtained from the Muroran Marine Station, Field Science Center for Northern Biosphere, Hokkaido University. The effect of artificial chelator, such as EDTA and HBED, on the maturation of gametophyte was also examined. Maturation of gametophyte was minor in the chelate-free medium, even in the higher concentration of Fe. In contrast, 70 – 80 % of gametophytes were matured in the presence of Fe and EDTA. However, the percent maturity was decreased with an increase in EDTA/Fe molar ratio from 1 to 100. These

results show that the uptake of Fe from the Fe-EDTA complex was inhibited in the presence of excess EDTA. These results suggest that the ligand-exchange reaction between EDTA and binding sites on the cell membrane is major process for taking Fe into the gametophyte. In the presence of Fe-HBED complex ($\log K_{\text{Fe(III)-HBED}} = 39$), which is more stable complex than EDTA ($\log K_{\text{Fe(III)-EDTA}} = 25$), no gametophyte were matured. This supports the mechanisms for Fe uptake into gametophyte *via* the ligand-exchange reaction. In the presence of Fe-SWEOM complex, 30 – 75 % of gametophytes were matured, suggesting that the Fe-SWEOM complex is bioavailable species to the maturation of gametophyte.

In conclusion, SWEOM can serve as a chelator to supply bioavailable Fe to seaweed in coastal seawater. The findings in the present study will contribute to better understanding of the reforestation processes of seaweed-bed by fertilizing dissolved Fe, as well as of the mechanistic statements for the technique of fertilization in barren coastal areas.

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