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Determination of Labile Fe(II) Species Complexed with Seawater Extractable Organic Matter Under Seawater Conditions Based on the Kinetics of Ligand-exchange Reactions with Ferrozine

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A fertilizer, comprised of a mixture of steel slag and compost, was used to restore seaweed beds in barren coastal areas. Complex Fe(II) species, supplied by steel slag, play a significant role in supplying Fe(II) to coastal areas and stimulating seaweed growth. Seawater extractable organic matter (SWEOM) from compost is generally assumed to serve as a chelator of Fe(II) in the fertilizer. It is considered that the bioavailability of Fe(II)-SWEOM complexes is higher in the dissociable (labile) species. In the present study, a method for determining labile species of Fe(II)-SWEOM complexes in seawater (pH 8.0, I = 0.7) was developed. The method is based on a ligand-exchange reaction between SWEOM and ferrozine (FZ). Because Fe(II) is readily oxidized to Fe(III) under normal seawater conditions, ascorbic acid was added as an antioxidant. The coloring for the Fe-FZ complex in the presence of SWEOM was retarded. This retarding can be attributed to a ligand-exchange reaction between FZ and labile Fe(II)-SWEOM complexes. Conditional binding constants for the labile Fe(II)-SWEOM complexes and binding capacities of labile sites in SWEOM to Fe(II) were evaluated for a variety of total Fe(II) concentrations.

Keywords Seawater extractable organic matter, iron, lability, complex, ferrozine, seawater, stability constant, binding capacity

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Introduction

Barren ground is a phenomenon associated with the depletion of seaweed in coastal areas. The development of barren ground has been attributed to a lack of soluble Fe (<1 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 area seawater can lead to seaweed depletion. It is known that natural 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 allows for its elution 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 complexes is a key factor in determining the bioavailability of metal species.[2,3,22,24,25] Readily dissociable complexes (labile species) are the likely primary source of soluble iron for algae.[2,19,26-28] 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,[17,29] although the conditions (pH 3.6 or 5, I = 0.02) were far from those for seawater (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 levels because Fe(II) is readily oxidized to insoluble Fe(III)-hydroxides, which precipitates from the solution.[30] Indeed, pH and ionic strength both have a dramatic

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effect on the speciation of dissolved metal ions in the presence of dissolved organic matter, such as humic acids.[11,31-35] 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.[36,37] 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 the present study, 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.

Experimental

Materials

Ammonium iron(II) sulfate hexahydrate ($Fe(SO_4)_2(NH_4)_2{\cdot}6H_2O$), 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). A supply of 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%).

Fractionation of SWEOM samples

SWEOM was extracted by artificial seawater from the bark compost, according to the method described in a previous study.[17,18] A mixture of compost and seawater (solid/liquid = 1:10, wt/wt) was shaken under a N_2 atmosphere for three 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 the 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 fractions were freeze-dried to obtain powdered SWEOM samples. Fractions with a molecular weight below 0.5 kDa were acidified to pH < 1.0 with concentrated HCl. The acidified fractions were passed through a DAX-8 resin, which is an acrylic-ester resin for the adsorbent of phenolic moieties in humic matter.[38] The adsorbed fractions were 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) and the acidic functional group content (carboxylic acids and phenolic hydroxyl groups) were determined, according to the procedures and conditions reported in a previous study.[18,39] To estimate the molecular size distribution, concentrations of total organic carbon (TOC) of collected fractions were analyzed by means of a TOC-V CSH analyzer (Shimadzu).

Analysis of labile Fe(II) species

Buffered seawater was prepared by dissolving the following salts in 1 L of ultrapure water: NaCl, 28.0; $MgSO_4{\cdot}7H_2O$, 7.0; $MgCl_2{\cdot}6H_2O$ 4.0; $CaCl_2$ 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 ASP_{12} medium that is typically used for culturing marine macroalgae.[4,5,40] 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 Fe^{2+} (2 mM) was prepared by dissolving $Fe(SO_4)_2(NH_4)_2{\cdot}6H_2O$ 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 Fe^{2+} 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.

Results and Discussion

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,[17] the following equilibrium can be written:

$$Fe^{2+} + \text{SWEOM} \rightleftharpoons \text{Fe(II)-SWEOM}. \quad (1)$$

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

$$Fe^{2+} + FZ \rightleftharpoons \text{Fe(II)-FZ}, \quad (2)$$

$$\text{Fe(II)-SWEOM}_{\text{labile}} + FZ \rightleftharpoons \text{SWEOM} + \text{Fe(II)-FZ}. \quad (3)$$

In Eq. (3), the Fe(II)-SWEOM complex can be regarded as a labile Fe(II) species complexed with SWEOM ($\text{Fe(II)-SWEOM}_{\text{labile}}$). 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. 1). This

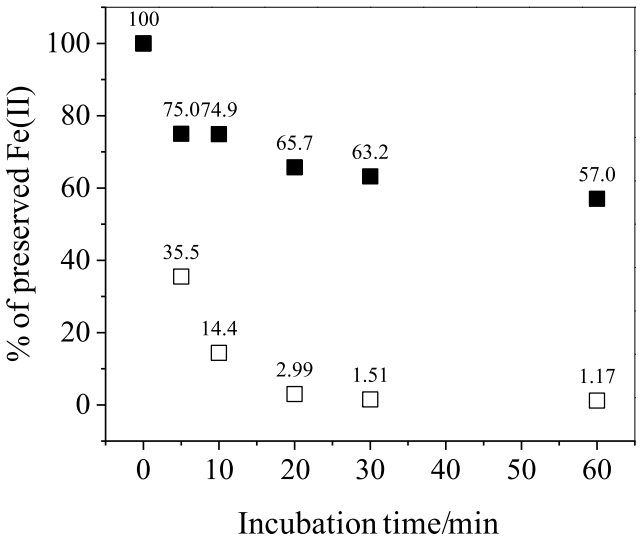

Fig. 1 Kinetics for the bleaching of color for Fe(II)-FZ complex. Closed and open squares denote reactions run in the presence (20 mM) and absence of ascorbic acid, respectively. Total injected Fe^{2+} concentration was 50 μM. Average values are labeled on each plot (S.D. = 0 – 2.1, n = 3).

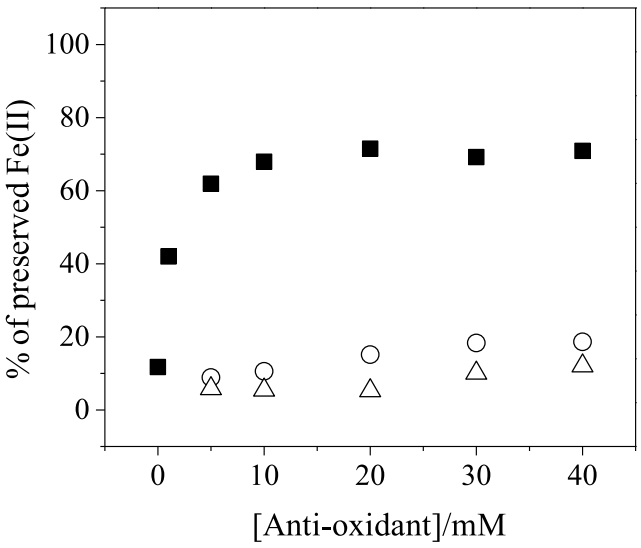

Fig. 2 Effect of anti-oxidant concentration on preserving Fe(II). Symbols: ■, ○ and △ represent ascorbic acid, hydroxylamine and glucose, respectively (S.D. = 0.1 – 4.4, n = 3). Total Fe^{2+} concentration was 50 μM. Incubation time from point of ferrozine addition was 30 min.

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 antioxidants was examined. Figure 2 shows the influence of antioxidant 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 for determining total Fe in seawater in a previous study.[37] As shown in Fig. 1 (■), the detectable Fe(II) species were preserved by ascorbic acid, even in seawater conditions. In addition, the percentage of preserved

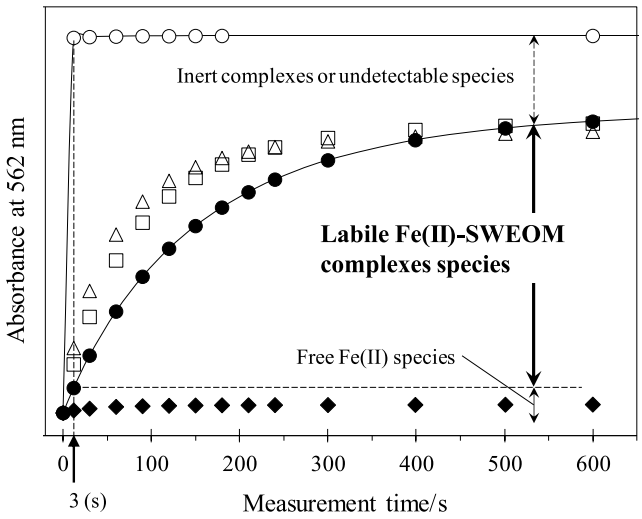

Fig. 3 Kinetics of the increase in absorbance at 562 nm as the result of forming Fe(II)-FZ complexes in the absence and the presence of SWEOM, and a schematic diagram for determining the free species of Fe(II) and labile Fe(II)-SWEOM complex species. Added Fe concentration was 10 μM. Symbols: ○ represents 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}).

Fe(II) reached plateau at ascorbic acid concentrations above 10 mM (Fig. 2). Approximately 30% of Fe was not detected as Fe(II), even under higher concentrations of ascorbic acid (Fig. 2). However, 90 – 95% of total Fe was detected as Fe(II)-ferrozine colored complex after 24 h. These results suggested that approximately 30% of Fe exists as Fe(III) during the analysis time (30 min).

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).[41] At 50 μM of 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 3 shows the kinetic curves for formation of the colored Fe(II)-FZ complex in the absence and presence of SWEOM. The complexation between free Fe(II) and FZ was a fast reaction for 25 μS that was detectable by means of a stopped-flow spectrophotometry (Fig. S1(A), Supporting Information). Although formation of the Fe(II)-FZ complex was detectable in the presence of SWEOM (Fig. S1(C)), the increase in Fe(II)-FZ formation, based on the ligand-exchange in Eq. (3), could not be detected by the stopped-flow spectrophotometry. Thus, the kinetic mode of the UV-vis spectrophotometer was employed in the present study. As shown in Fig. 3 ○, absorbance value at 3 s was used for the estimation of free Fe(II) concentrations because this point is the limitation for detection of the UV-vis spectrophotometer. The absorbance values for the plateau region were proportional to the prepared Fe(II) concentration, indicating that the absorbance values in the absence of SWEOM can be used for the purpose of calibration. However, in the

presence of SWEOM (Fig. 3, ●, □ 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 higher SWEOM concentrations. It is generally assumed that humic substances contain strong and weak binding sites.[27,32,42-44] The strong sites in SWEOM would be occupied first, with the formation of kinetically stable complexes.[27,42] 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 trace amounts of iron and this may affect the kinetic curves in Fig. 3. Thus, iron in SWEOM samples were determined by FZ colorimetry after agitating the solution with ascorbic acid for 900 s. It was found that NMW, HMW and LMW contained 18, 36 and 8 μmol g^{-1} of Fe, respectively. The kinetic curve for SWEOM without Fe addition (Fig. 3, ◆) showed that the effect of initial iron in the original sample of SWEOM is negligible.

As shown in Fig. 3, 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. 3), 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.

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

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.[42] Accordingly, the complexing abilities of size fractionated SWEOMs should be examined. To determination 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:

$$TOC_{0.5\ kDa>} = TOC_{>100\ kDa} \times \frac{Yield_{0.5\ kDa>}}{Yield_{>100\ kDa}}, \quad (4)$$

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, 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

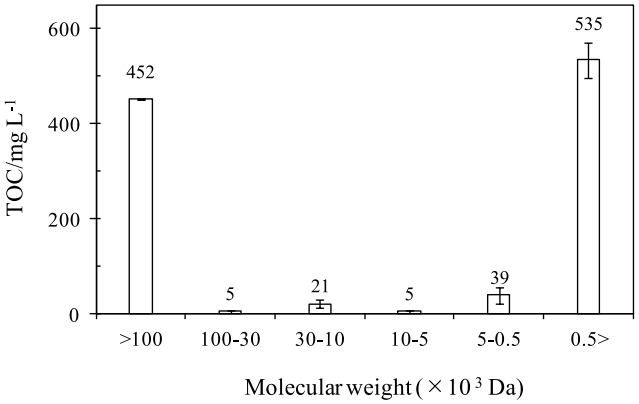

Fig. 4 Distribution of molecular weight fractions in the SWEOM.

contents for NMW, HMW and LMW are summarized in Table S1 (Supporting Information). The elemental compositions of NMW and HMW were similar, supporting the results shown in Fig. 4 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 S1). These results indicate that alcohols, ethers and esters are the major oxygen-containing functional groups in NMW and HMW.

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

$$Fe^{2+} + SWEOM \rightleftharpoons Fe(II)\text{-}SWEOM_{labile}. \quad (5)$$

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

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

The total concentration of binding sites for forming labile Fe(II)-SWEOM complexes (C_L) can be expressed as:

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

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

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

K_b and C_L were calculated by the non-linear least square regression analysis of the data set for $[Fe(II)]_{free}$ and $[Fe(II)\text{-}SWEOM]_{labile}$ to Eq. (8).[29,31] The binding capacity (BC) can also be calculated as:

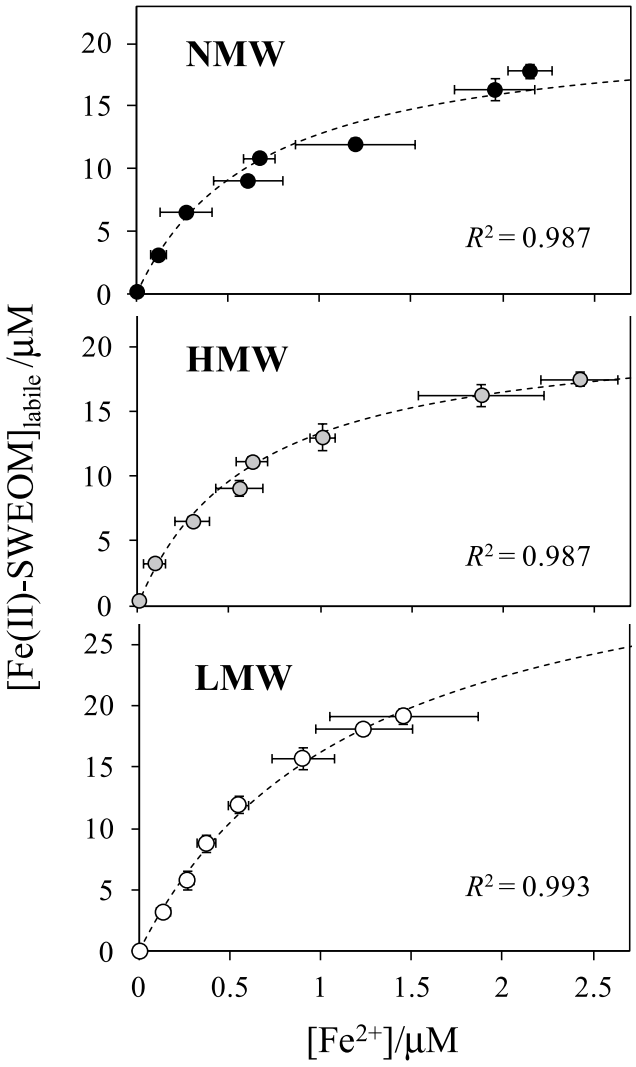

Fig. 5 Relationships between Fe(II) and labile Fe(II)-SWEOM concentrations at [SWEOM] 50 mg L^{-1}: NMW (a), HMW (b) and LMW (c). Error bars represent standard deviations (n = 4 – 5).

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

where the concentration of SWEOM was 0.05 g L^{-1}, and the $\%C$ is the carbon content in Table S1. Figure 5 shows plots and fitting curves for the three fractions. The total Fe(II) concentrations varied from 0 to 28 μM. The experimental data sets were well fitted to Eq. (8) with the square of the correlation coefficients (R^2 in Table 1) 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 the major amino acid residues in SWEOM.[18] In this model experiment, glutamic acid and total Fe(II) concentrations were 100 and 0 to 32 μM, 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 the IUPAC stability constants database (5.13 at 20°C and I = 0.65).[45] The C_L value indicates that glutamic acid forms the dimeric complexes with Fe(II). These results suggest that the method proposed in the present study is useful for the determination of conditional stability constant for Fe(II)-dissolved organic matter complexes under seawater conditions.

Table 1 Conditional binding constants (log K_b) for labile Fe(II)-SWEOM species and binding capacities (BC) of SWEOM to Fe(II)

Fraction[a]	log K_b	BC (μmol g^{-1} 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.

The log K_b and BC values for NMW, HMW and LMW are summarized in Table 1. The values for log K_b and BC for NMW and HMW were similar, which is 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.[14,29,33] 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 S1). Based on the HSBA principle, oxygen-containing groups are classified as hard bases,[14,46] and Fe(II) is classified as a borderline acid.[47] However, nitrogen-containing groups can be classified as a borderline base that has a strong affinity to Fe(II). As indicated in Table 1, 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 S1), 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.

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 re-oxidation 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.

Acknowledgements

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Supporting Information

This material is available free of charge on the web at http://www.jsac.or.jp/analsci/.

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