Effects of Chelated Iron on the Growth of Sargassaceae Species at the Germling and Immature Stages

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ABSTRACT
Sargassaceae species are important macroalgae for maintaining marine environments. Furthermore, biofuel based on Sargassaceae species has become studied recently. However, Sargassaceae species have declined rapidly in coastal areas around Japan, which are called "isoyake", a term meaning barren areas. The development of effective measures to restore these "isoyake" and the construction of Sargassaceae beds are strongly needed. A lack of iron in coastal areas limits the growth of macroalgae, and may cause "isoyake". However, the effects of the iron concentration on the growth of macroalgae, such as species of Sargassaceae, are poorly studied. In this research, the effects of chelated iron on the growth of Sargassaceae species during the germling and immature stages were examined. The addition of Fe-EDTA promoted the growth of four Sargassaceae species during the germling stages. During the germling stage of *Sargassum ringgoldianum*, the maximum specific growth rate and the saturation constant were estimated to be 0.17 day^{-1} and 4.3 μg/L, respectively. Additionally, the periodic addition of Fe-EDTA promoted the stable growth of *Sargassum horneri* during the immature stage.

Keywords: chelated iron, growth, Sargassaceae species

INTRODUCTION

Over 60 known Sargassaceae species are widely distributed in coastal areas around Japan. Sargassaceae species form marine forests, which provide marine habitats, take up nutrients, and absorb dissolved carbon dioxide and thus, play an important role in marine environments. Furthermore, biofuel production from Sargassaceae species of fuels such as bioethanol has been recently studied. However, seaweeds, including species of Sargassaceae, have declined rapidly in coastal areas around Japan, which are now called "isoyake", a term meaning barren areas. The development of effective measures to restore these "isoyake" and the reconstruction of seaweed beds are of immediate concern. The reasons for the rapid extension of "isoyake" are not well understood. The loss of seaweeds, such as species of Sargassaceae, is caused by a rise in the sea temperature, a decrease of nutrients, a decrease in salinity, and the intensive grazing pressures of herbivorous marine animals (Fujita *et al*., 2010). Many research groups have reported the effects of temperature, irradiance, and the length of day on the growth of Sargassaceae species (Hwang *et al*., 2006; Choi *et al*., 2008; Pang *et al*., 2009). However, the effects of iron have not received as much attention in "isoyake" research.

Iron availability limits the growth of algae, and its deficiency has been shown to limit the growth of phytoplankton (Martin and Fitzwater, 1988). In seawater, iron is slightly dissolved using a natural iron-chelating agent such as fulvic acid. The decrease of chelated iron in coastal waters causes a decrease in macroalgae (Matsunaga, 2003). A few reports have examined the effects of chelated iron on the growth of macroalgae. For

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example, previous studies investigated the effects of iron ethylenediaminetetraacetic acid (Fe-EDTA) on oogenesis and gametogenesis in *Laminaria angustata* (Motomura and Sakai, 1981, 1984), the uptake rate of Fe-EDTA by the adult macroalgae *Laminaria religiosa Miyabe* and *Undaria pinnatifida* (Matsunaga *et al*., 1991), and the effects of Fe-EDTA on the oogenesis and vegetative growth of kelp gametophytes (*Phaeophyceae*) (Lewis *et al*., 2013). A study of the effects of Fe-EDTA on the photosynthetic pigments and ultrastructure of chloroplasts in *Porphyra yezoensis* showed that the lack of Fe-EDTA caused "chlorosis" in the thalli of *Porphyra yezoensis*, suggesting that the synthesis of photosynthetic pigments was depressed without the addition of Fe-EDTA (Ueki *et al*., 2010). The application of humic substances as a natural iron-chelating agent on iron elution from steelmaking slag has been studied. Mixing the steelmaking slag and humic substances allowed the iron to elute from the steelmaking slag (Yamamoto *et al*., 2010, 2011). Mesocosm experiments monitored the effect of mixing steelmaking slag and humic substances as a potential fertilizer source for the growth of *Nori* (Ueki *et al*., 2011).

However, we are not aware of any reports of the effects of the addition of chelated iron on the growth of Sargassaceae species during the germling and immature stages. In this study, the effects of Fe-EDTA addition on the growth of four Sargassaceae species (three *Sargassum* and one *Myagropsis*) during the germling and immature stages were examined. The objective of this study is to clarify whether Fe-EDTA addition can control the growth of four Sargassaceae species. Additionally, this study attempts to discover the Fe-EDTA concentration needed for promoting the growth of Sargassaceae species during the germling and immature stages.

MATERIALS AND METHODS

Fertilized eggs from female plants of four Sargassaceae species were collected from Fukui Pref., Japan. *Myagropsis myagroides, Sargassum horneri, Sargassum patens,* and *Sargassum ringgoldianum* were collected in 2012 on April 25, May 8, May 29, and September 19, respectively. Fertilized eggs were rinsed with filtered seawater, poured into amber glass bottles with filtered seawater, and refrigerated. Figure 1 shows the fertilized eggs of the four collected Sargassaceae species. Seawater was sampled from the deep ocean of Noto Peninsula in Ishikawa Pref., Japan. Table 1 shows the pH and nutrient concentrations of the filtered seawater. Table 2 shows the nutrient contents of 1 mL of PES (Enriched Seawater by Provasoli) media (Ariga *et al*., 2000), which was used to make enriched seawater. Enriched seawater was prepared by adding 20 mL of PES media to 1000 mL of filtered seawater, resulting in 11.8 mg-N/L, 1.48 mg-P/L, and 550 μg-Fe/L.

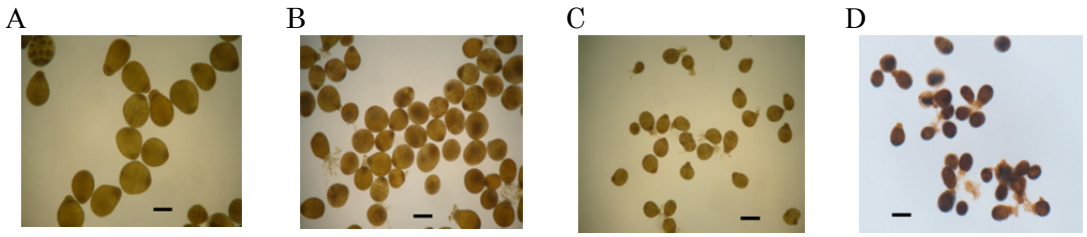

Fig. 1 - Fertilized eggs of four Sargassaceae species. A: *Myagropsis myagroides*, B: *Sargassum horneri*, C: *Sargassum patens*, and D: *Sargassum ringgoldianum*. Scale bar: 200 μm.

Table 1 - Values of pH, nutrient concentrations, and Fe concentrations of seawater from Noto Peninsula.

pH (-)	NH_4-N (mg/L)	NO_2-N (mg/L)	NO_3-N (mg/L)	PO_4-P (mg/L)	Fe (μg/L)
8.16	0.03	< 0.01	0.24	0.036	< 0.5

Table 2 - Nutrient and Fe contents of 1 mL of PES media.

$NaNO_3$ (mg)	Na_2 glycerophosphate (mg)	Fe (μg)	EDTA (mg)
3.5	0.5	27.5	0.417

Effect of adding Fe-EDTA on the growth of Sargassaceae species during the germling stage

Figure 2 shows the photo-incubators (LTI-700, EYELA, Japan) with LED lights (LDR14N-W, TOSHIBA, Japan). The photo-incubators were maintained at 80 – 100 μmol photons/m^2/s and 20°C, with a 12:12 h light:dark cycle. Light intensities were measured using a light quantum meter (MQ-200, Apogee Instruments, USA). Two experiments by using the photo-incubator A were performed to investigate the effect of chelated iron addition on the growth of Sargassaceae species during the germling stage.

The first experiment cultivated *Myagropsis myagroides*, *Sargassum horneri*, and *Sargassum patens* in enriched seawater with or without Fe-EDTA. The purpose of the first experiment was to confirm that the Fe-EDTA addition can promote the growth of Sargassaceae species during the germling stage. After sterilization, 30 mL of filtered seawater was poured into two culture dishes (diameter = 8.7 cm). Either 0.6 mL of PES media or 0.6 mL of Fe-removed PES media was added to the culture dish. Thus, primary Fe-EDTA concentrations were controlled at 0 μg/L and 550 μg/L, respectively. The fertilized eggs were separated into the culture dishes using pipettes (15 eggs per dish) and cultivated for 20 days.

The second experiment cultivated *Sargassum ringgoldianum* in enriched seawater with five different Fe-EDTA concentrations. The purpose of the second experiment was to estimate the Fe-EDTA concentration needed for the growth of Sargassaceae species during the germling stage. After sterilization, 30 mL of filtered seawater was poured into five culture dishes (diameter = 8.7 cm). All culture dishes were established with 0.6

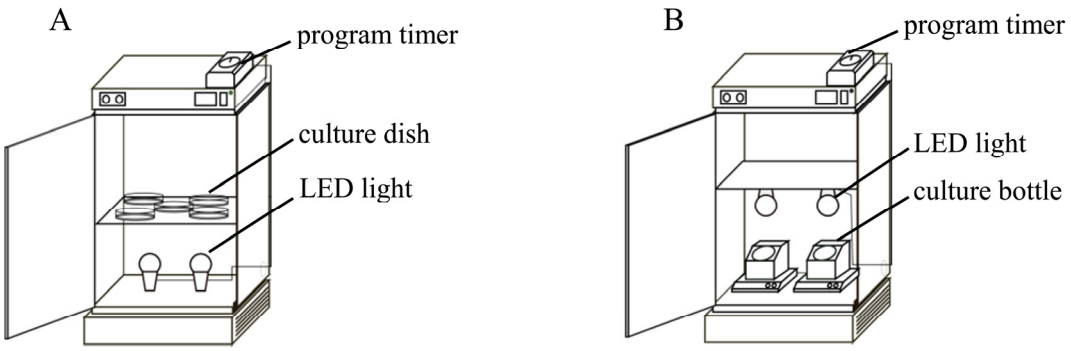

Fig. 2 - Photo-incubator A containing five culture dishes used to cultivate four Sargassaceae species during the germling stages, and photo-incubator B containing two culture bottles used to cultivate *Sargassum horneri* during the immature stage.

mL of Fe-removed PES media. Each dish received 0.1 mL of a solution, each of which contained a different Fe-EDTA concentration. The Fe-EDTA concentrations of the five dishes were 0, 5, 20, 50, and 500 μg/L. The Fe-EDTA concentrations, except for 500 μg/L, were determined according to the dissolved iron concentrations of coastal areas in Japan, as listed in a reference book (Okaichi, 2000). The fertilized eggs were separated into the culture dishes using pipettes (15 eggs per dish) and cultivated for 30 days.

The growth of Sargassaceae species was observed using a stereoscopic microscope (SMZ745T, Nikon, Japan) mounted with a camera (DS-Fi2-L3, Nikon, Japan). The algae body was analyzed by image analysis software (ImageJ, NIH). Some of the fertilized eggs, which were dead during the cultivation, were removed from the data. Obtaining the area of the algae body allowed the specific growth rate (μ) to be calculated using the following equation:

$$\mu = (\ln A_{t2} - \ln A_{t1}) / (t_2 - t_1) \quad (1)$$

where t_1 is the initial time (day) of the logarithmic growth phase, t_2 is the final time (day) of the logarithmic growth phase, A_{t1} is the area (mm^2) on the initial day of the logarithmic growth phase, and A_{t2} is the area (mm^2) on the final day of the logarithmic growth phase.

Effect of adding Fe-EDTA on the growth of *Sargassum horneri* during the immature stage

The effect of adding Fe-EDTA on the growth of *Sargassum horneri* during the immature stage was investigated by using the photo-incubator B shown in Fig. 2. *Sargassum horneri* was cultivated in enriched seawater with or without Fe-EDTA. After sterilization, 600 mL of filtered seawater was poured into two polycarbonate culture bottles (CB-2B, AZONE). Each culture bottle received 12 mL of either PES media or Fe-removed PES media. The Fe-EDTA concentrations were 0 μg/L and 550 μg/L. The medium was renewed once per week. Three immature specimens of *Sargassum horneri* within the nets were separated into two culture bottles (three immature specimens per bottle) and cultivated for 42 days. The culture bottles were continuously stirred at 500

rpm. The fresh weights of the three immature specimens of *Sargassum horneri* were recorded once per week.

ANALYTICAL METHODS

Seawater analysis

Seawater was filtered through a membrane filter (0.45-μm pore size). The pH, dissolved nitrogen concentration, and phosphorus concentration were analyzed according to JISK 0102 (Japanese Standards Association, 2008). Dissolved Fe was analyzed using inductively coupled plasma mass spectrometry with a cool plasma condition after solid-phase chelate extraction (Aimoto *et al*., 2010).

Chlorophyll analysis

The concentrations of chlorophylls *a*, *b*, and *c* were determined by the method presented in Jeffrey and Humphrey (1975). The thalli were transferred to a solution of 90% acetone and degraded by ultrasonic disintegration. The suspension was centrifuged at 3,000 rpm for 10 min (H-19α, KOKUSAN, Japan). The absorbance of the supernatant at 664 nm, 647 nm, and 630 nm was measured using a spectrophotometer (U-2910, HITACHI, Japan). The chlorophyll *a*, *b*, and *c* contents per dry weight were calculated using Eqs. 2, 3, and 4, respectively.

$$\text{Chl } a = 11.85 \times \text{A664} - 1.54 \times \text{A647} - 0.08 \times \text{A630} \quad (2)$$

$$\text{Chl } b = -5.43 \times \text{A664} - 21.03 \times \text{A647} - 2.66 \times \text{A630} \quad (3)$$

$$\text{Chl } c1+c2 = -1.67 \times \text{A664} - 7.06 \times \text{A647} + 24.52 \times \text{A630} \quad (4)$$

RESULTS AND DISCUSSION

Effect of adding Fe-EDTA on the growth of Sargassaceae species during the germling stage

Figure 3 shows the growth curves of three Sargassaceae species in enriched seawater with and without the addition of Fe-EDTA. All species showed an increase in thalli area when Fe-EDTA was added at a concentration of 550 μg/L. After 20 days of cultivation, the thalli areas with the addition of Fe-EDTA were 2 to 10 times larger than those without Fe-EDTA added. From these results, the added 550 μg/L of Fe-EDTA promoted the growth of Sargassaceae sp. during the germling stage. Figure 4 shows the growth curves and thallus elongations (after 30 days) of *Sargassum ringgoldianum* in enriched seawater with the five different Fe-EDTA concentrations. In the case of *Sargassum ringgoldianum,* the addition of Fe-EDTA promoted the growth of thalli during the germling stage, as noted for other Sargassaceae species. In addition, from the growth rate curves of Figure 4, the specific growth rate of *Sargassum ringgoldianum* tended to depend on Fe-EDTA concentrations. Thus, the specific growth rate of *Sargassum ringgoldianum* could be estimated with different Fe-EDTA concentrations.

Figure 5 (left) shows the relationship between the Fe-EDTA concentration and the specific growth rate of *Sargassum ringgoldianum* during the germling stage. Assuming that the specific growth rate of *Sargassum ringgoldianum* increases in accordance with the Michaelis-Menten equation, the equation can be expressed as follows:

$$\mu = \mu_{max} (S / (S + Ks)) \quad (5)$$

where μ (day^{-1}) is the specific growth rate, μ_{max} (day^{-1}) is the maximum specific growth rate, Ks (μg/L) is the saturation constant, and S (μg/L) is the Fe-EDTA concentration. In addition, by plotting the reciprocal of μ and S, which is called the Lineweaver-Burk plot, the two factors of μ_{max} and Ks can be estimated. Figure 5 (right) shows the Lineweaver-Burk plot. From Figure 5, μ_{max} and Ks were estimated to be 0.17 day^{-1} and 4.3 μg/L during the germling stage, respectively.

These experimental results clarified that chelated iron such as Fe-EDTA was indispensable for the growth of Sargassaceae species at the germling stage. In addition, it was estimated that the Fe-EDTA concentration needed for the growth of *Sargassum ringgoldianum* during the germling stage was 4.3 μg/L as the saturation constant. In addition, this saturation constant value is considered to correspond reasonably well with the reported Fe concentrations of coastal areas (Okaichi , 2000).

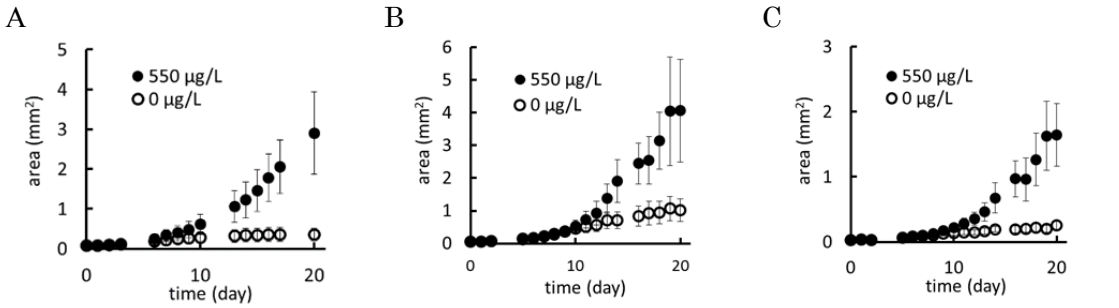

Fig. 3 - Growth curves of the mean area ± the standard error for three Sargassaceae species in enriched seawater with and without Fe-EDTA added (A: *Myagropsis myagroides*, B: *Sargassum horneri*, C: *Sargassum patens*). The number of samples was n = 15 for A, n = 14 for B, and n = 13 for C.

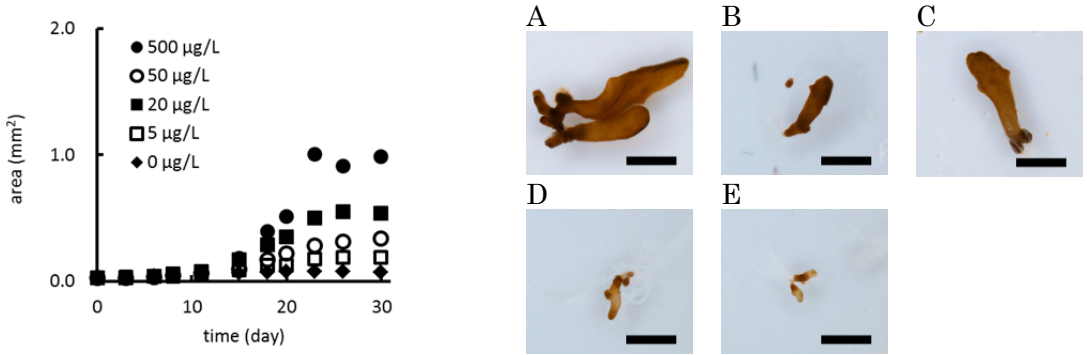

Fig. 4 - Growth curves of the mean areas of *Sargassum ringgoldianum* in enriched seawater with five Fe-EDTA concentrations and thallus elongations after 30 days with different Fe-EDTA additions (A: 500 μg/L, B: 50 μg/L, C: 20 μg/L, D: 5 μg/L, E: 0 μg/L Fe-EDTA addition). The number of samples was n = 10 for A, n = 4 for B, n = 6 for C, n = 4 for D, and n=8 for E. Scale bar: 1,000 μm.

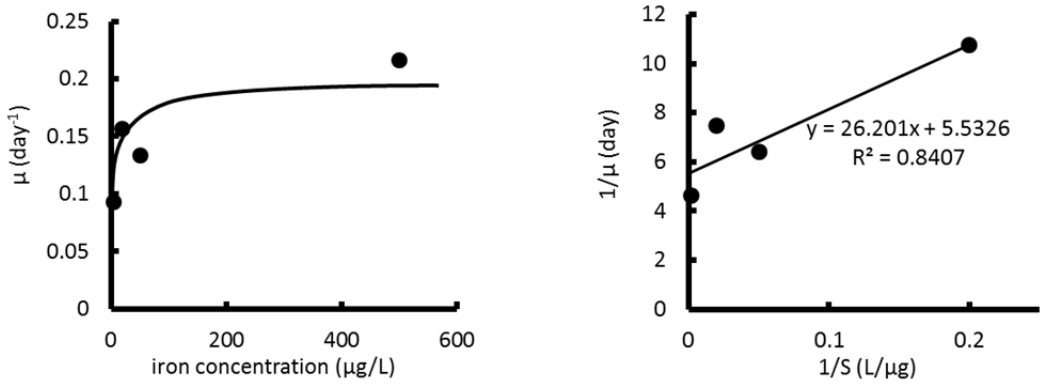

Fig. 5 - Relationship between the added Fe-EDTA concentrations and the specific growth rate of *Sargassum ringgoldianum* during the germling stage (left). The Lineweaver-Burk plot is on the right.

Effect of adding Fe-EDTA on the growth of *Sargassum horneri* during the immature stage

Figure 6 shows the growth curves of *Sargassum horneri* in enriched seawater with and without Fe-EDTA added during the immature stage. When 550 μg/L of Fe-EDTA was added, the wet weight of *Sargassum horneri* tended to continuously increase for 42 days. Without the addition of Fe-EDTA, the wet weight of *Sargassum horneri* tended to decrease after 21 days. The thalli started to be destroyed after 32 days, and two of the three samples of *Sargassum horneri* appeared to die 10 days later. Figure 7 shows the comparison of the absorption spectra of the thalli cultured in enriched seawater between samples with and without Fe-EDTA added. Both thalli strongly showed absorbance at 660 nm and 430 nm, which indicated the presence of chlorophyll *a*. The concentration of chlorophyll *a* in thalli with Fe-EDTA added was 220 μg/g-dry weight. The chlorophyll *a* content in thalli without Fe-EDTA added was 160 μg/g-dry weight. As shown in Fig. 7, the thalli without Fe-EDTA added appeared to be in chlorosis because of the lack of chlorophyll *a*. The synthesis of photosynthetic pigments was estimated to be depressed without periodic Fe-EDTA addition.

These experiments clearly proved that even if there were plenty of nitrogen and phosphorus in seawater, the lack of chelated iron can limit the growth of Sargassaceae species at both the germling and immature stages. Thus, it is concluded that the lack of chelated iron will contribute to “isoyake” phenomena, along with other reasons. However, it is considered that the chelated iron concentrations needed for many other Sargassaceae species should be continuously studied and, thus, the chelated iron concentrations needed for Sargassaceae species must be more precisely clarified. Moreover, instead of Fe-EDTA, chelated irons using natural iron-chelating agents such as humic substances should be examined. These fundamental studies for the growth of Sargassaceae species using chelated irons are considered to give important knowledge for the practical restoration of “isoyake” phenomena in coastal areas.

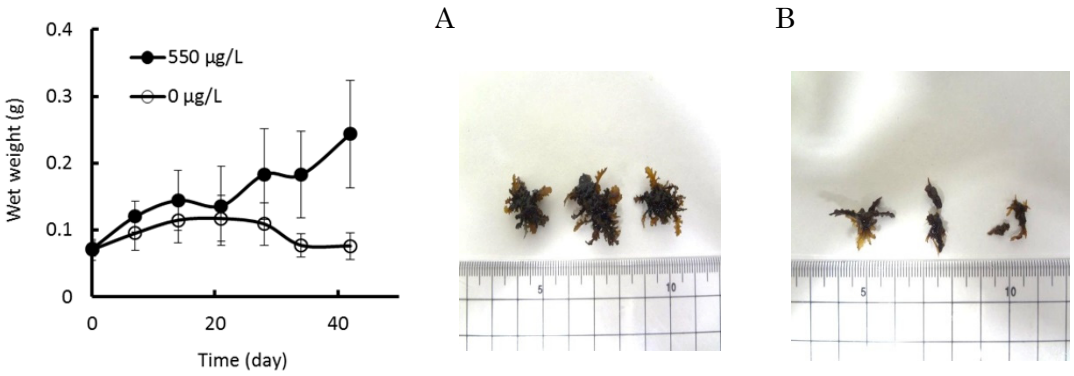

Fig. 6 - Growth curves of the mean wet weight ± the standard error for *Sargassum horneri* in enriched seawater with and without Fe-EDTA added. The number of samples was n = 3 for A and B (left). Thallus elongations after 42 days with different Fe-EDTA additions (A: 550 µg/L of Fe-EDTA addition, B: no Fe-EDTA addition) (right).

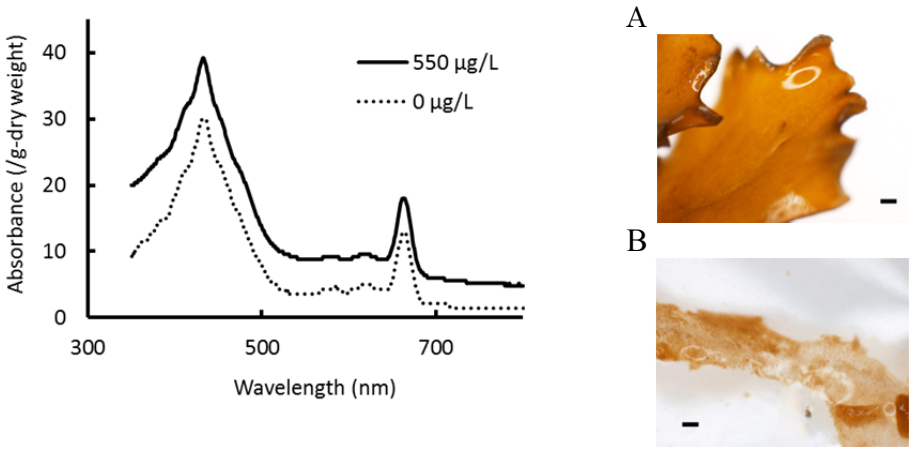

Fig. 7 - Comparison of the absorption spectra of thalli cultured in enriched seawater between *Sargassum horneri* samples with and without Fe-EDTA added (left). Comparison of thalli after 42 days between *Sargassum horneri* samples with and without Fe-EDTA added (A: 550 µg/L, B: no Fe-EDTA addition) (right). Scale bar: 200 µm.

CONCLUSIONS

The effects of chelated iron (Fe-EDTA) on the growth of four Sargassaceae species during the germling and immature stages were examined. The addition of Fe-EDTA promoted the growth of four Sargassaceae species during the germling stage. The overall maximum specific growth rate and the saturation constant were estimated to be 0.17 day^{-1} and 4.3 µg/L, respectively, during the germling stage of *Sargassum ringgoldianum*. The periodic addition of Fe-EDTA promoted the stable growth of *Sargassum horneri* during the immature stage. The thalli cultivated without Fe-EDTA appeared to be in chlorosis because of a lack of chlorophyll *a*. The synthesis of photosynthetic pigments was estimated to be depressed without Fe-EDTA addition. It is

concluded that the addition of Fe-EDTA clearly affects the growth of many Sargassaceae species during the germling and immature stages. Additionally, the need for more research using other naturally chelated irons instead of those chelated with EDTA is recognized.

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