Effects of Fe fertilizer eluate on the growth of Sargassum horneri at the germling and immature stages

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Effects of Fe-fertilizer Eluate on the Growth of *Sargassum horneri* at the Germling and Immature Stages

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Abstract

To aid in the restoration of coastal barren ground areas, it is important to clarify the effects of chelated iron on the growth of seaweed. In particular, for the further development of practical methods to promote seaweed growth, Fe-binding organic ligands, such as humic substances (HSs) composed of humus materials, rather than Fe-binding inorganic ligands, such as ethylenediaminetetraacetic acid (EDTA), should be investigated. In this study, the effects of an Fe-fertilizer, made from HSs and steelmaking slag, on the growth of *Sargassum horneri* at the germling and immature stages were examined. The addition of the Fe-fertilizer eluate containing Fe-organic ligand complexes clearly promoted the growth of *Sargassum horneri* at the germling and immature stages. It was also clear that the effect of Fe concentration in the Fe-fertilizer eluate on the growth rate was almost the same as that of Fe-EDTA. Moreover, the addition of the Fe-fertilizer eluate had a great effect on the brown color of *Sargassum horneri* thalli and promoted the increased content of photosynthetic pigments, such as Chl. *a*. Based on these experimental results, the application of the Fe-fertilizer containing Fe-organic ligand complexes is expected to become an effective method for the restoration of the barren ground phenomenon in Fe-deficient coastal areas.

Keywords Fe-fertilizer · Chelated iron · Growth · *Sargassum horneri* · Humic substance

Introduction

Seaweed beds composed of many types of macroalgae and a creature group comprised ecosystems that are important to the fishery economy and conservation of coastal environments. However, seaweed has declined rapidly resulting in barren grounds in many coastal areas around Japan (Fujita *et al*., 2010). In Japan, such barren grounds are referred to as "isoyake." The rapid decrease of seaweed beds in coastal areas is considered to have various complex causes: rising sea temperatures, decrease of nutrients, decrease in salinity, and intensive grazing pressures of herbivorous marine animals (Fujita *et al*., 2010; Choi *et al*., 2008; Hwang *et al*., 2006; Pang *et al.,* 2009). Various measures to prevent the isoyake phenomenon have been undertaken to protect marine resources such as seaweed.

The iron concentration in the ocean affects the growth of algae, and insufficient iron concentrations have been found to limit the growth of phytoplankton (Martin and Fitzwater, 1988; Turner and Hunter, 2001). The decrease in iron concentration in coastal areas has been reported as an important contributing factor to the isoyake phenomenon around Japan (Matsunaga *et al*., 1991; Matsunaga, 2003). However, compared to nitrogen or phosphate deficiency, less attention has been paid to iron deficiency as an important limiting nutrient factor for the isoyake phenomenon.

Several fundamental studies have been conducted to clarify the effects of chelated iron on seaweed growth. Ethylenediaminetetraacetic acid (EDTA) is widely used as an artificial chelating agent in lab-scale experiments to maintain iron in a dissolved condition in seawater (Provasoli, 1968). It has been reported that most female gametophytes in *Saccharina angustata* formed oogonia at 2.0 mg L^{-1} of Fe-EDTA (Motomura and Sakai, 1981). The gametogenesis of *Saccharina* and *Desmarestia* were induced in a medium containing a high concentration of Fe-EDTA (Motomura and Sakai, 1984). The Ks (half-saturation constant) values for *Saccharina religiosa* and *Undaria pinnatifida* were 54 and 98 nM, respectively, in the presence of Fe-EDTA (Matsunaga *et al*., 1991). The lack of Fe-EDTA caused chlorosis in the thalli of *Pyropia yezoensis*, suggesting that the synthesis of photosynthetic pigments was depressed

without the addition of Fe-EDTA (Ueki *et al*., 2010). The addition of Fe-EDTA promoted the growth of four Sargassaceae species during the germling stages, and the maximum specific growth rate and the saturation constant of *Sargassum ringgoldianum* were estimated to be 0.17 day^{-1} and 4.3 μg L^{-1}, respectively (Nagai *et al*., 2014).

Recently, from the perspective of practical use, the application of humic substances (HSs) as natural Fe-chelating agents rather than EDTA has been studied because artificial chelating agents such as EDTA cannot be used in the actual marine environment. For example, a Fe-fertilizer comprising HSs and steelmaking slag has been developed to supply dissolved iron to coastal areas. The Fe-fertilizer eluate contains high concentrations of dissolved iron, and it has been reported that field experiments using the Fe-fertilizer in Hokkaido showed that *Saccharina* seaweed beds had expanded (Yamamoto *et al*., 2006, 2010, and 2011). Mesocosm experiments using the Fe-fertilizer clarified that it was effective for promoting the growth of *P. yezoensis*, *Saccharina japonica* (Ueki *et al*., 2011, 2012, Kato *et al*., 2014). However, even though Sargassaceae are an important species in the marine forest, there are few studies about the effects of the Fe-fertilizer on the growth of Sargassaceae species during the germling and immature stages. In particular, *Sargassum horneri* is one of Sargassaceae species distributed widely in the northwest Pacific coasts and has been paid much attention for the restoration of seaweed beds (Choi *et al*., 2008, Pang *et al*., 2009). So, this paper focuses on *S. horneri*.

This study aimed to clarify the effects of the eluate from the Fe-fertilizer on the growth of *S. horneri*, particularly during the germling and immature stages. The effects of the Fe-fertilizer eluate on thallus color and the amount of photosynthetic pigments, such as chlorophyll, were investigated.

Materials and methods

Sargassum horneri

S. horneri was used to evaluate the effects of Fe-fertilizer eluate on growth during the germling and immature stages. Fertilized eggs from the female plants of *S. horneri* were collected from Fukui Prefecture, Japan, in April 2013 and 2014. After gathering, the fertilized eggs were rinsed with filtrated seawater, poured into amber glass bottles (500 mL) with filtrated seawater, and preserved in a refrigerator at 4°C until use.

Culture medium and analysis of seawater

The seawater used for the culture medium of *S. horneri* was sampled using a suction pump at a depth of 320 m in the deep ocean off the Noto Peninsula, north of the Ishikawa Prefecture. Deep seawater (DSW)-intake facilities have been constructed in Uchiura-machi of the Ishikawa Prefecture. The culture of macroalgae using DSW has been studied for the features of cleanness, low temperature and much nutrients (Fujita *et al*., 2006, Ueki *et al*., 2012). The seawater was filtrated through a membrane filter (0.45 μm pore size) before culture. Filtrated seawater quality was analyzed to determine pH, inorganic nitrogen (NH_4–N, NO_2–N, and NO_3–N), phosphate (PO_4–P), and iron (Fe) content. The pH was measured using a pH meter (HM-25R, DKK-TOA Corp., Japan). Nitrogen and phosphate concentrations were determined by a colorimetric method using an auto analyzer (Auto Analyzer, TRAACS2000, Bran Luebbe, Germany). Fe concentration was determined using a trace iron analyzer (FEA-07, Kimoto Electric Corp., Japan) based on luminol chemiluminescence. Filtrated seawater quality is shown in Table 1. Fe was not detected in the seawater (concentration limit for iron detection: 0.5 μg L^{-1}), which indicates that Fe is deficient in the seawater off the Noto Peninsula.

Fe-fertilizer eluate

A Fe-fertilizer was prepared with a steelmaking slag and humus material (1:1 weight ratio, Fig. 1). Steelmaking slag contains approximately 20 weight % Fe. Fulvic acids (FAs) eluted from the humus materials were expected to produce natural chelated iron as iron fulvate. The Fe-fertilizer eluate was manufactured as follows. Filtrated seawater was sterilized by autoclaving at 121°C for 20 min (SN-200, Yamato, Japan). After sterilization, 900 mL of the filtrated seawater was added to a polyethylene bottle (2 L). 300 g of the Fe-fertilizer was added to the bottle. Nitrogen gas was used to purge air from the bottle, and the bottle was sealed under anaerobic conditions. The bottle was placed in the dark at room temperature for 9 days with continuous agitation at 100 rpm using a magnetic stirrer. After 9 days, the supernatant in the bottle was filtrated through a No. 5A filter (47 mm diameter, 7 μm pore size, Advantec, Japan). The filtrated seawater was used as the Fe-fertilizer eluate. The chemical composition of the Fe-fertilizer eluate is shown in Table 2. Fe concentration of the Fe-fertilizer eluate was 320 μg-Fe L^{-1}, which seemed to be produced by Fe-binding organic ligands such as fulvate. And, dissolved organic carbon (DOC) concentration of the Fe-fertilizer eluate was 48.5 mg L^{-1}, which indicated containing organic ligands such as fulvate. DOC concentration of the Fe-fertilizer eluate was determined using TOC analyzer (TOC-VCPH, Shimadzu Corp., Japan)

S. horneri culture experiments at the germling stage

Filtrated seawater was sterilized by autoclaving at 121°C for 20 min (SN-200, Yamato, Japan). After sterilization, 30 mL of filtrated seawater was poured in ten culture dishes (diameter = 8.7 cm). All culture dishes were established with 0.6 mL of Fe-removed PES media (Provasoli, 1968; Motomura, 2000). Fe concentrations were controlled as follows. One dish had no Fe added and was the control dish. Three dishes received EDTA solutions. The Fe-EDTA concentrations in the three dishes were 5, 15, and 30

μg-Fe L^{-1}, and these were classified as Fe-EDTA dishes (Table 3). Next, three dishes received 0.5, 1.5, and 3.0 mL of the Fe-fertilizer eluate (Fe concentrations: 5, 15, and 30 μg-Fe L^{-1}). These were classified as Fe-fertilizer eluate dishes (Table 3).

The fertilized eggs were transferred from the amber glass bottles to the culture dishes using pipettes (15 eggs per dish) and were cultured for 18 days in photoincubators (EYELA LTI-700, Okyo Rikakikai Co., Ltd., Japan). Figure 2 (A) shows the photoincubators with LED lights (LDR14N-W, Toshiba, Japan). The photoincubators were maintained at 80–100 μmol photons m^{-2} s^{-1} and 20°C with a 12 h:12 h light:dark cycle. Light intensities were measured using a light quantum meter (MQ-200, Apogee Instruments, U.S.A.).

The growth of *S. horneri* was observed using a stereoscopic microscope (SMZ745T, Nikon, Japan) mounted with a digital camera (DS-Fi2-L3, Nikon, Japan). The area of the algae body was analyzed by image analysis software (ImageJ, NIH). The fertilized eggs that were found to be dead during the culture experiments were removed and excluded from the data.

The specific growth rate (μ) with the area of the algae body was calculated using the following equation:

$$\mu = \frac{\ln A_{t_2} - \ln A_{t_1}}{t_2 - t_1} \quad (1)$$

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.

The maximum specific growth rate (μ_{max}) and the saturation constant (Ks) based on the area of the algae body were estimated by using Lineweaver–Burk plot (Nagai *et al.*, 2014).

S. horneri culture experiments at the immature stage

After sterilization, 200 mL of filtrated seawater was poured in three glass culture bottles (250 mL). All culture bottles were established with 4.0 mL of Fe-removed PES media (Provasoli, 1968; Ariga *et al*., 2000). Fe concentrations were controlled as follows. One culture bottle with no Fe added was used as the control bottle. The second culture bottle received EDTA solutions (Fe concentration, 15μg-Fe L^{-1}; the Fe-EDTA bottle) (Table 3). The third culture bottle received 10.0 mL of the Fe eluate (Fe concentration, 15 μg-Fe L^{-1}; Fe-fertilizer eluate bottle) (Table 3).

Four *S. horneri* samples cultured with the enriched seawater containing 2% PES (Fe-EDTA: 550 μg-Fe L^{-1}), which were about 6.0 mg (wet weight), were transferred to the three glass bottles and were cultured for 42 days at a low aeration (0.2 mL min^{-1}) in photoincubators (SLC-25A, Mitsubishi Electric Engineering, Japan). Figure 2 (B) shows the photoincubators with LED lights (SLED-F30D, Japan global, Japan). The photoincubators were maintained at 80–100 μmol photons m^{-2} s^{-1} and 20°C with a 12 h:12 h light:dark cycle. The media in the three bottles was renewed once per week, and the wet weight of four samples from each culture bottle was measured.

After 42 days, the photosynthetic pigment content of *S. horneri* of each culture bottle was analyzed. The freeze-dried thalli of *S. horneri* 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 663, 645, and 470 nm was measured using a spectrophotometer (U-2910, Hitachi, Japan). The concentrations of photosynthetic pigment contents were determined using the methods presented by Porra (2002). Chl. *a*, Chl. *b*, and carotenoids content on a dry weight basis was calculated using Eqs. 2, 3, and 4, respectively.

$$\text{Chl.}\,a = 12.21 \times A_{663} - 2.81 \times A_{645} \quad (2)$$

$$\text{Chl.}\,b = 20.13 \times A_{645} - 5.03 \times A_{663} \quad (3)$$

$$\text{Carotenoids} = \frac{1000 \times A_{470} - 3.27 \times [\text{Chl.}\,a] - 104 \times [\text{Chl.}\,b]}{227} \quad (4)$$

Results

Germling stage experiments

Figure 3 shows the growth curves of *S. horneri* in the enriched seawater with and without two types of chelated Fe added. From these results, the addition of Fe-EDTA or the Fe-fertilizer eluate clearly promoted the growth of *S. horneri* during the germling stage. Figure 4 shows the relationship between the specific growth rates (μ) of *S. horneri* and added Fe concentrations. The μ of *S. horneri* with two types of Fe added tended to increase in the same way. The μ of Fe-EDTA and the Fe-fertilizer eluate were almost same. The μ were 0.3, 0.4, and 0.4 day^{-1} for 5, 15, and 30 μg-Fe L^{-1}, respectively.

The maximum specific growth rates (μ_{max}) and saturation constants (Ks) of *S. horneri* with two types of Fe added were estimated using the Lineweaver–Burk plot (Nagai *et al.*, 2014). For Fe-EDTA, μ_{max} and Ks were estimated to be 0.44 day^{-1} and 2.35 μg-Fe L^{-1}. For the Fe-fertilizer eluate, μ_{max} and Ks were estimated to be 0.41 day^{-1} and 2.10 μg-Fe L^{-1}. There was not a clear difference of μ_{max} and Ks at the germling stage between Fe-EDTA and the Fe-fertilizer. The data presented in this experiment show that Fe-organic ligand complexes contained in the Fe-fertilizer eluate and Fe-EDTA are effective for promoting the growth of *S. horneri* at the germling stage.

The comparison of thalli color between *S. horneri* cultured for 18 days with two different types of Fe added, as well as the growth rates under each condition, is shown in Fig. 5. The addition of Fe-EDTA or the Fe-fertilizer eluate clearly affected the thalli color of *S. horneri* during the germling stage. The brown color of thalli deepened as the Fe concentration increased. The thalli of *S. horneri* cultured in the 5 μg-Fe

L^{-1} addition exhibited slight chlorosis. The thalli of *S. horneri* cultured in the 15 and 30 μg-Fe L^{-1} additions clearly deepened the brown color of the thalli. The comparison of the photosynthetic pigment content of the thalli between *S. horneri* samples cultured for 18 days with the two types of added Fe is shown in Fig. 6. Chl. *a* content values cultured using Fe-EDTA or the Fe-fertilizer eluate clearly depended on the increase of Fe concentrations. Compared with Chl. *a*, the effect of Fe concentration on carotenoid content was unclear. Carotenoid content calculated by Eq. 3 may be easily affected by Chl. *a* content values. Further testing is needed to confirm carotenoid content precisely. When comparing samples grown in the presence of Fe-EDTA or the Fe-fertilizer eluate, the photosynthetic pigment content values cultured using the Fe-fertilizer eluate were slightly higher than those cultured using Fe-EDTA. However, further study will be required to confirm the difference between Fe-EDTA and the Fe-fertilizer eluate. These results show that Fe-fertilizer eluate addition can deepen the brown thallus color and increase photosynthetic pigment content, such as Chl. *a*, during the germling stage.

Immature stage experiments

Figure 7 shows the growth curves of the wet weights of *S. horneri* cultured for 42 days with three different Fe additions during the immature stage. Figure 8 shows the comparison of thalli between *S. horneri* cultured for 42 days under three Fe addition conditions. In the case of Fe-EDTA or the Fe-fertilizer eluate addition (15 μg-Fe L^{-1}), the wet weight of *S. horneri* continued to increase during the 42 days. There was not a clear difference between Fe-EDTA and the Fe-fertilizer for *S. horneri* growth during the immature stage. However, without Fe added, the wet weight of *S. horneri* did not increase, and the thalli of *S. horneri* were thin and easily teared. From these results, the periodical addition of the Fe-fertilizer eluate, as well as Fe-EDTA, is effective for promoting the growth of *S. horneri* during the immature stage.

The comparison of the photosynthetic pigment content of the thalli between *S. horneri* samples cultured for 42 days without and with the two types of Fe added is shown in Fig. 9. When comparing samples grown in the presence of Fe, Chl. *a* content values in the thalli of *S. horneri* cultured using Fe-EDTA or the Fe-fertilizer eluate tended to be greater than for those cultured without Fe addition. The effect of Fe concentration on carotenoid content during the immature stage as well as during the germling stage was not clear. When comparing samples grown in the presence of Fe-EDTA versus the Fe-fertilizer eluate, there was not a clear difference in the photosynthetic pigment content. As shown in Fig. 8, the brown thallus color after 42 days was maintained in the presence of Fe-EDTA and the Fe-fertilizer eluate. From these results, the Fe-fertilizer eluate can increase the Chl. *a* contents of thalli and deepen the brown thallus color during the immature stage.

Discussion

These experiments clearly show that *S. horneri* can grow definitely using Fe-organic ligands compounds in the Fe-fertilizer eluate as well as an Fe-inorganic ligand compound using Fe-EDTA. With regard to Fe concentration, the effects of Fe-organic ligand compounds in the Fe-fertilizer eluate on the growth rate of *S. horneri* at the germling and immature stages are almost as same as those of an Fe-inorganic ligand compound using EDTA. It has been reported that 0.2 μM (11.2 μg-Fe L^{-1}) of Fe-EDTA and Fe-FA are effective for oogonium formation, growth rate, and pigment synthesis of *Saccharina japonica* (Suzuki *et al*., 1994, 1994, and 1995; Matsunaga, 2003). The effect of Fe concentrations on the growth rate of *Saccharina japonica* in the past study (Suzuki *et al*., 1994) was almost the same as that of *S. horneri* in the present study. There does not appear to be a clear difference between Fe-EDTA and the Fe-fertilizer with regard to appropriate Fe concentration to promote the growth of macroalgae at the germling and immature stage.

In a sense, this is an important result because it was expected that it would be more difficult for macroalgae to grow when the more complicated Fe-organic ligands compounds in the Fe-fertilizer eluate were used than when Fe-inorganic ligand compounds with EDTA were used. The chemical speciation of Fe-organic ligand compounds in the Fe-fertilizer eluate has not been clarified in this study. The Fe-organic ligand compounds in the Fe-fertilizer eluate may have chemical forms similar to Fe-FA found in previous studies (Suzuki *et al*., 1994, 1994, and 1995; Matsunaga, 2003). Moreover, the Fe-fertilizer eluate is estimated to contain particulate forms of Fe-compounds <7 μm. Bioavailable forms of Fe-organic ligand compounds absorbed by Sargassaceae species should be studied more precisely.

In addition to the effects on the growth rate, it was clarified that the Fe-fertilizer eluate deepens the brown thalli color and increases the concentration of photosynthetic pigments, such as Chl. *a*, in the thalli of *S. horneri*. It has been suggested that this result for *S. horneri* was also found in the case of chlorosis in the thalli of *Pyropia yezoensis* without the addition of Fe-EDTA (Ueki *et al*., 2010). Fe-organic ligands compounds in the Fe-eluate, as well as Fe-inorganic ligand compounds using EDTA, can be utilized for the synthesis of photosynthetic pigments.

Based on these experimental results, the use of the Fe-fertilizer produced by mixing humus materials and steelmaking slag, rather than using chemical agents such as EDTA, is expected to become a practical method for the restoration of Sargassaceae species in the barren ground of Fe-deficient coastal areas. Studies using such Fe-fertilizer rather than EDTA will expand the fundamental knowledge of the bioavailable mechanisms of Fe to promote the growth of macroalgae and will lead to practical applications to restore barren ground areas. However, the chemical speciation of Fe-organic ligands compounds and the bioavailable forms of Fe-organic ligands compounds in the Fe-fertilizer eluate were not clear and should be investigated further. In addition, determining the duration of the eluate of dissolved Fe-organic ligand compounds in the Fe-fertilizer applied to coastal areas will be important. Moreover, the effects of Fe-organic ligand compounds in the Fe-fertilizer eluate on HAB (Harmful algal

bloom) remains to be investigated. Further research is in progress to clarify these problems. Continuing studies for the use of the Fe-fertilizer to algae will yield further insight into the isoyake phenomenon and an effective method for the restoration of the barren ground phenomenon in Fe-deficient coastal areas.

Conclusion

The effects of the Fe-fertilizer eluate containing Fe-organic ligand complexes on the growth of *S. horneri* at the germling and immature stages were examined. The addition of the Fe-fertilizer eluate clearly promoted the growth of *S. horneri* at both the germling and immature stages. It was shown that there was not a large difference between the effects of Fe concentration of the Fe-fertilizer eluate and that of Fe-EDTA. Moreover, the addition of the Fe-fertilizer eluate had a stronger effect on the color of *S. horneri* thalli and increased the amount of photosynthetic pigments, such as Chl. *a*.

Therefore, it is concluded that the addition of the Fe-fertilizer eluate produced by mixing humus materials and steelmaking slag is effective for improving growth, deepening the thallus color, and increasing the concentration of photosynthetic pigments during the germling and immature stages for *S. horneri*. The application of the Fe-fertilizer to Fe-deficient coastal areas is expected to become an effective method for the restoration of the barren ground phenomenon. In addition, the chemical and bioavailable forms of Fe-organic ligand complexes, including particulate forms of Fe, on the growth of Sargassaceae species should be studied more precisely. More detailed studies will contribute to a better understanding of the isoyake phenomenon in Fe-deficient coastal areas.

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Table 1 Nutrient levels of the seawater from Noto peninsula

pH	NH_4–N	NO_2–N	NO_3–N	Inorganic–N	PO_4–P	Fe
(-)	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(μg L^{-1})
8.6	<0.01	<0.01	0.29	0.29	0.052	<0.5

Table 2 Nutrient levels of the Fe-fertilizer eluate

pH	NH_4–N	NO_2–N	NO_3–N	Inorganic–N	PO_4–P	Fe
(-)	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(μg L^{-1})
8.1	3.74	8.23	21.15	33.1	0.54	320

Table 3 Nutrient levels of each culture media

	NH_4–N	NO_2–N	NO_3–N	Inorganic–N	PO_4–P	Fe
	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(μg L^{-1})
Control	<0.01	<0.01	11.6	11.6	1.04	<0.5
Fe-EDTA	<0.01	<0.01	11.6	11.6	1.04	5, 15, 30
Fe-fertilizer	0.06	0.13	12.2	12.4	1.04	5
	0.18	0.39	12.9	13.4	1.02	15
	0.34	0.75	13.8	14.9	1.00	30

Figure captions:

Fig. 1 Fe-fertilizer and a process for manufacturing Fe-fertilizer eluate

Fig. 2 Photoincubators for culturing *S. horneri* during the germling (A) and immature (B) stages

Fig. 3 Growth curves of the mean thalli area of *S. horneri* cultured in the enriched seawater with and without two types of added Fe. Values are the means of fifteen replicates±SD (A: Fe-EDTA; B: the Fe-fertilizer eluate)

Fig. 4 Relationship between the specific growth rates of *S. horneri* and added Fe concentrations during the germling stage (A); Lineweaver–Burk plot (B)

Fig. 5 Comparison of thalli color between *S. horneri* samples cultured for 18 days with two types of added Fe (A, B, and C: Fe-EDTA addition at 5, 15, and 30 μg-Fe L^{-1}, respectively; D, E, and F: Fe-fertilizer eluate addition at 5, 15, and 30 μg-Fe L^{-1}, respectively). Scale bar: 1 mm

Fig. 6 Comparison of photosynthetic pigment content of thalli between *S. horneri* samples cultured for 18 days with two types of Fe added (A: Chl. *a*; B: carotenoids)

Fig. 7 Growth curves of the mean wet weight for *S. horneri* cultured in enriched seawater with and without two types of added Fe (Control: Fe addition at 0 μg-Fe L^{-1}; Fe-EDTA:Fe-EDTA addition at 15μg-Fe L^{-1}; Fe-fertilizer:Fe-fertilizer eluate addition at 15 μg-Fe L^{-1}). Values are the means of four replicates ±SD

Fig. 8 Comparison of *S. horneri* samples cultured for 42 days without and with two types of added Fe (A: Fe-fertilizer eluate addition at 15 μg-Fe L^{-1}; B: Fe-EDTA addition at 15μg-Fe L^{-1}; and C: Fe addition at 0 μg-Fe L^{-1}) Scale bar: 1 mm

Fig. 9 Comparison of photosynthetic pigment content of thalli among *S. horneri* of four samples cultured for 42 days without and with two types of added Fe

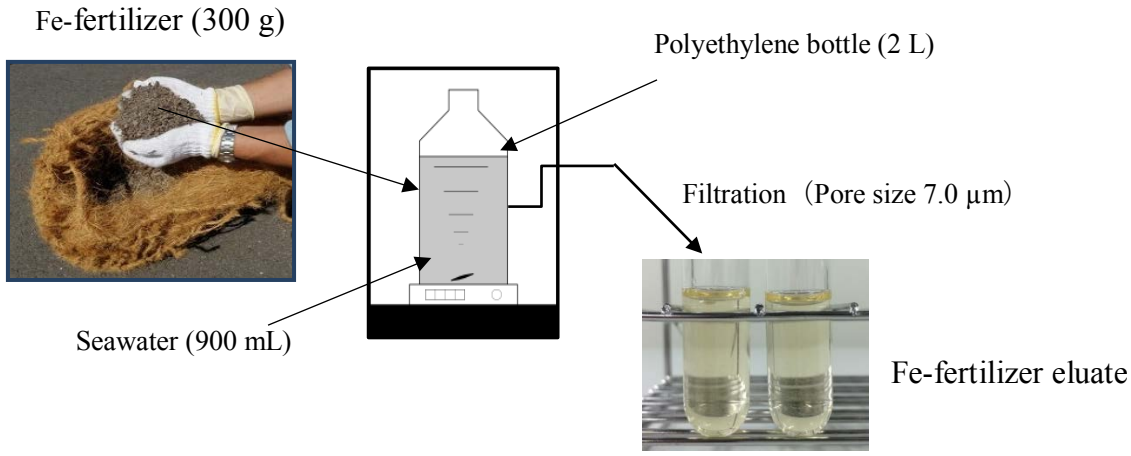
Fe-fertilizer (300 g)
Polyethylene bottle (2 L)
Filtration（Pore size 7.0 μm）
Seawater (900 mL)
Fe-fertilizer eluate

Fig. 1

(A) (B)

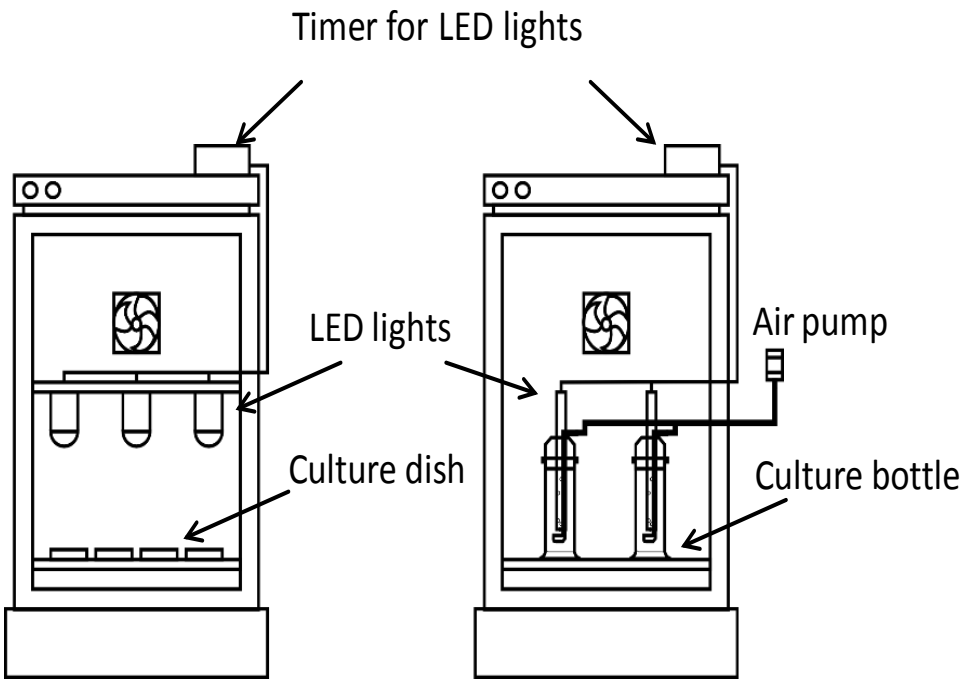
Timer for LED lights
LED lights
Culture dish
Air pump
Culture bottle

Fig. 2

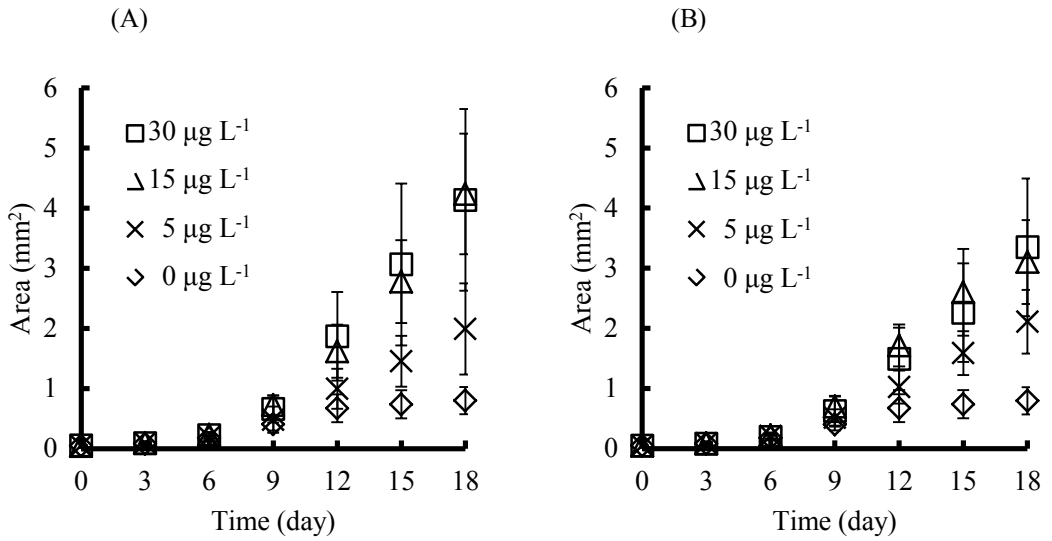
(A)
(B)
Area (mm²)
Time (day)
30 μg L-1
15 μg L-1
5 μg L-1
0 μg L-1
0
1
2
3
4
5
6
0
3
6
9
12
15
18

Fig. 3

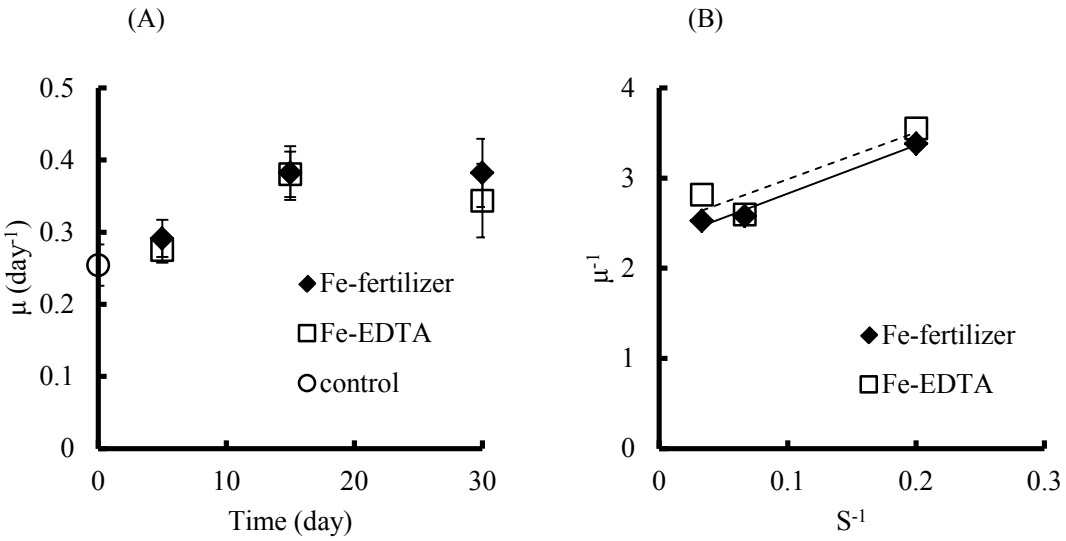
(A)
(B)
0.5
0.4
0.3
0.2
0.1
0
μ (day-1)
0
10
20
30
Time (day)
Fe-fertilizer
Fe-EDTA
control
4
3
2
1
0
μ-1
0
0.1
0.2
0.3
S-1
Fe-fertilizer
Fe-EDTA

Fig. 4

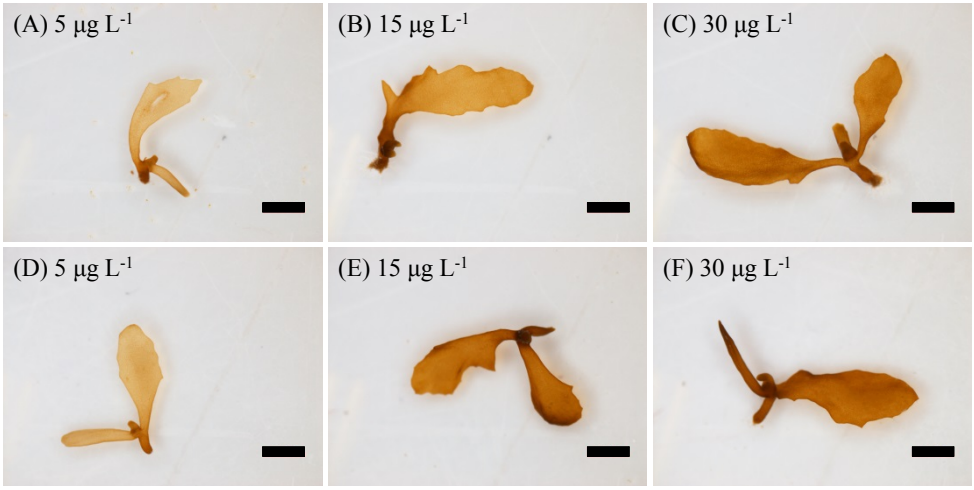

Fig. 5

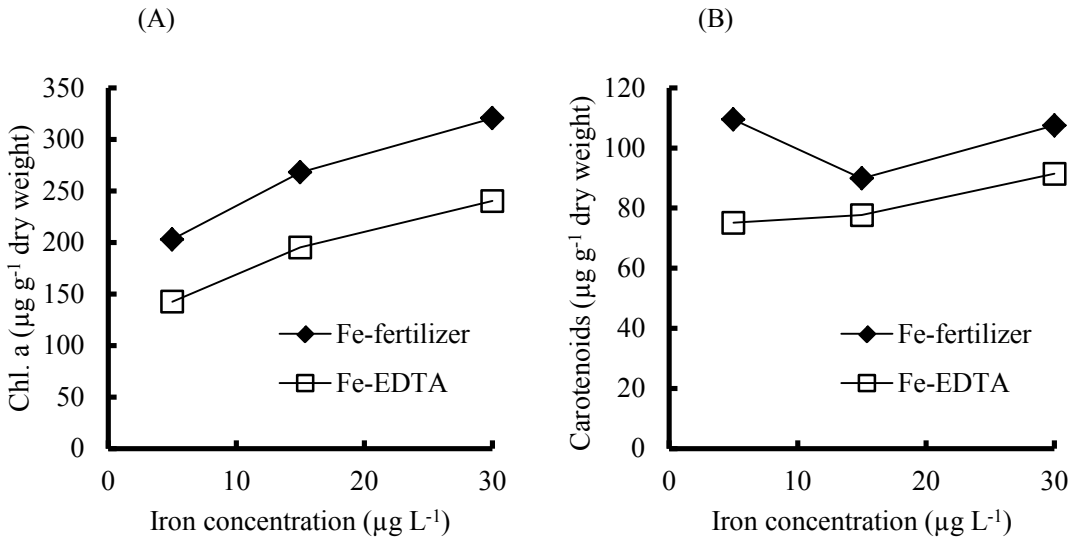
(A)
(B)
Chl. a (µg g-1 dry weight)
Carotenoids (µg g-1 dry weight)
Iron concentration (µg L-1)
Iron concentration (µg L-1)
Fe-fertilizer
Fe-EDTA
Fe-fertilizer
Fe-EDTA
0
50
100
150
200
250
300
350
0
20
40
60
80
100
120
0
10
20
30
0
10
20
30

Fig. 6

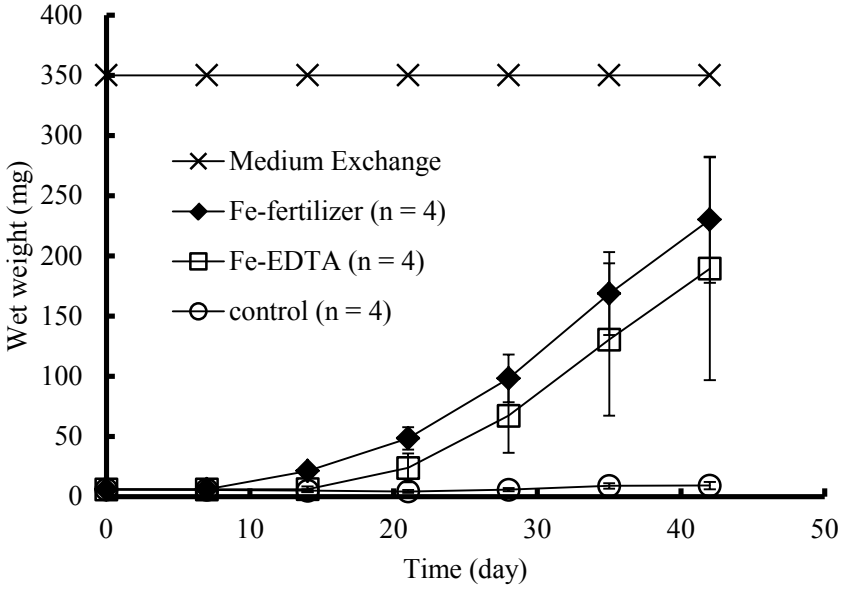
400
350
300
250
200
150
100
50
0
Wet weight (mg)
Medium Exchange
Fe-fertilizer (n = 4)
Fe-EDTA (n = 4)
control (n = 4)
0
10
20
30
40
50
Time (day)

Fig. 7

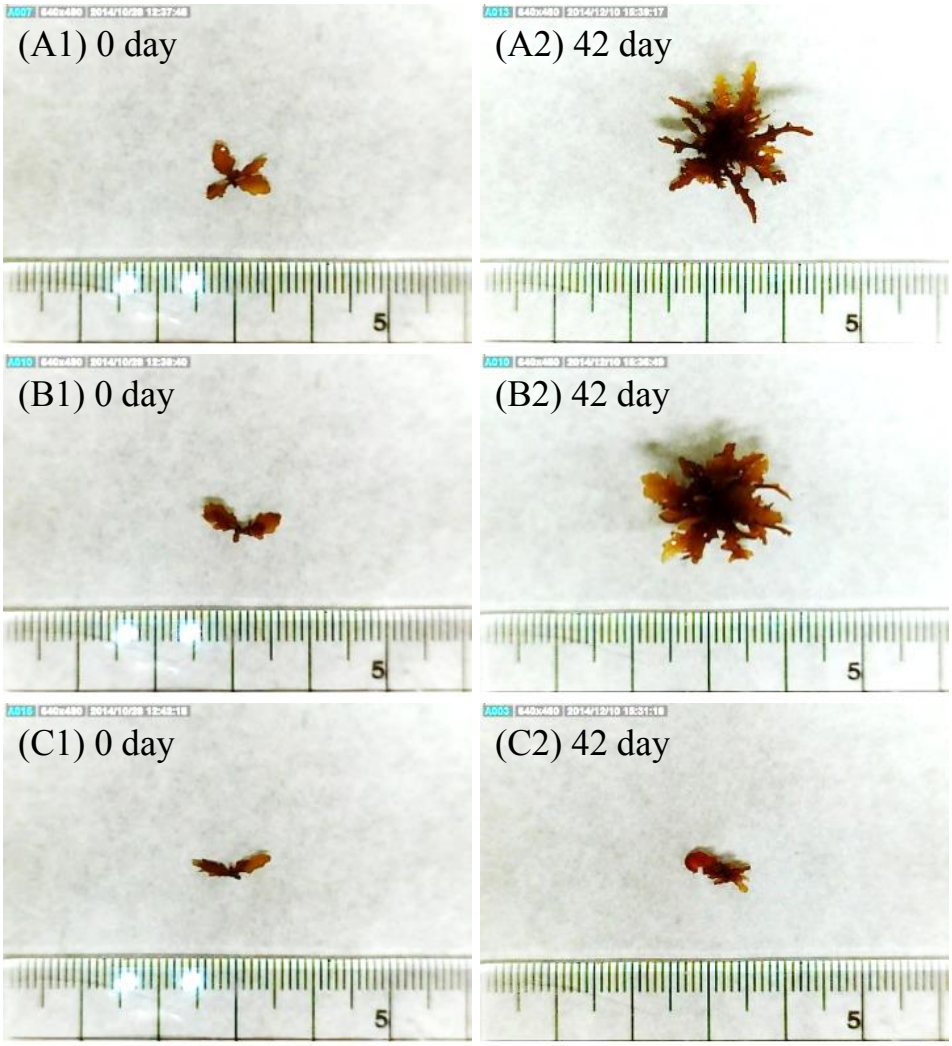

Fig. 8

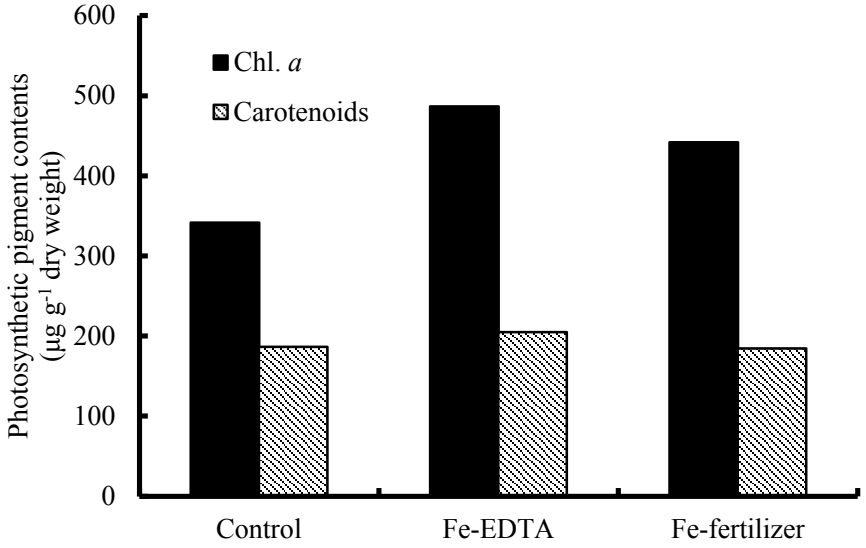
600
500
400
300
200
100
0
Photosynthetic pigment contents
(μg g-1 dry weight)
Chl. a
Carotenoids
Control
Fe-EDTA
Fe-fertilizer

Fig. 9