1. Introduction

Global warming is a looming environmental problem as temperature increase in the past century was reported to be 0.85°C over the period 1880 to 2012. Annual increments in CO₂ emission have proven to be the major cause of the global warming trend as evidenced by a correspondence between the increase/decrease in CO₂ concentration in the atmosphere and the increase/decrease in temperature. According to the Kyoto Protocol, Japan had to reduce CO₂ by six percent based on the 1990 level by 2012. Under the Clean Development Mechanism outlined in the Kyoto Protocol, developed countries were also required to invest and develop technologies for reducing CO₂ emission in developing countries as an alternative to more expensive emission reductions in their own countries.

Nuclear accidents are some of the major technological risks that modern societies have to face in their search for the most optimal energy solutions. Since the 1979 Three Mile Island (TMI) accident in USA to the most recent 2011 Fukushima core meltdown in Japan, there has been an ongoing debate on the use of nuclear energy. Indeed, Japan’s Fukushima nuclear disaster has encouraged the Japanese people to reconsider the use of nuclear energy and to search for a safer and renewable type of energy. A simultaneous solution to the problem of both global warming and getting a safe renewable energy might reside on the use of biofuels, which are environmentally friendly, fossil energy independent and carbon neutral. In particular, microalgae have been proposed as the perfect biofuel candidates because they can grow faster than plants and high-lipid content, do not require fertile land or useful water, reach solar energy utilization efficiencies of up to 5%, and are able to use directly exhaust gases as their carbon source.

In particular, the marine diatom Skeletonema costatum is a promising choice for biofuel production in terms of total oil yield producing 87%, the highest reported among oil crops and marine microalgae. S. costatum possess a desirable fatty acid profile, especially triglycerides, which make them perfect candidates for biodiesel production. Since this study has proven slag to be efficient in the enhancement of S. costatum growth, we believe this work will provide many benefits for the development of phytoplankton as biofuels in the near future.

Iron is known to be a limiting factor for the growth of phytoplankton in the open ocean, especially in HNLC (High Nutrient Low Chlorophyll) areas and also in the coastal waters. Therefore, a balanced nutrient supply is required for effective marine phytoplankton growth. Iron
slag and steel-making slag are by-products of the iron and steel manufacturing processes. These by-products are roughly classified into two types: blast furnace slag which is produced from the conversion process of iron ore into pig iron,\textsuperscript{18} and steel-making slag which is produced from the purification process of pig iron into steel. In 2010, a total of 40 MT of slag was generated in Japan, and 14 MT out of the total is steel-making slag.\textsuperscript{19} Steel-making slag contains silicon, phosphorus and iron, and may elute these elements as dissolved nutrients, \textit{i.e.}, silicate, phosphate and divalent/trivalent iron ions, which are essential for the growth of phytoplankton.\textsuperscript{20} However, since steel-making slag does not contain any nitrogenous compound, it will not be a nitrogen source. The dissolution behavior of silicate and phosphate from steel-making slag has only been tested recently.\textsuperscript{21} However, to date there are no known reports testing the dissolution behavior of iron in different chemical forms. Iron dissolution from slag into seawater is affected by a lot of chemical processes such as oxidation, chelation and hydrolysis. Thus, iron speciation is a very important factor in investigating the effect of iron dissolved from slag on the growth of marine phytoplankton.

This study is intended to clarify the dissolution behavior of iron species as well as dissolved inorganic phosphorus (DIP) and dissolved silicon (DSi) from steel-making slag for the first time. Moreover, it examines the effects of these dissolved elements on the growth of the marine diatom \textit{Skeletonema costatum} in comparison to other iron sources. This work will be of great value towards the practical application of steel-making slag as a cost-effective fertilizer for the mass production of microalgae as biofuels.

2. Materials and Methods

2.1. Dephosphorized Slag

The dephosphorized slag used in this study was provided by JFE Steel Corporation, Okayama, Japan. Dephosphorized slag is a type of steel-making slag produced from the dephosphorization process of steel. The phosphorus (P) content in dephosphorized slag appear to be higher than that in other slags, and a slight dissolution of phosphate from the slag can maintain the growth of phytoplankton.\textsuperscript{20} In the previous study,\textsuperscript{20} they confirmed appropriate amount of the slag for growth of phytoplankton using natural community corrected from Kure Bay, Hiroshima; 100 mg l$^{-1}$ was appropriate to enhance the growth, while 50 mg l$^{-1}$ was not sufficient for growth, and 500 mg l$^{-1}$ was judged to be high because growth was ceased due to high pH. Table 1 shows the chemical composition of the slag used in this study that is the same as used in the previous study.\textsuperscript{20} The concentrations of environmentally regulated substances in the slag were obviously below the detection limits.\textsuperscript{20}

2.2. Dissolution Behavior

2.2.1. Dissolution Test

Dephosphorized slag used in the dissolution test was passed through a sieve with the mesh size of 250 $\mu$m to make the size homogeneous. Artificial seawater (3% salinity) was prepared according to Keller \textit{et al.} (1987)\textsuperscript{22} and its chemical composition is shown in Table 2. Trace metals and micro nutrients were added to the artificial seawater following the method of Guillard and Hargraves (1993).\textsuperscript{23} However, phosphate, silicate and iron were not added to the artificial seawater in order to investigate their dissolution behavior from the steel-making slag. Thereafter, 2 L of artificial seawater were sterilized by filtering through a 0.22 $\mu$m membrane filter (Sterivex-GV, Millipore) on a clean bench and dispensed into a UV-sterilized Erlenmeyer flask. Next, 200 mg of UV-sterilized steel-making slag was added to the seawater and the flask was capped with a bacteria-free silicon cap to allow gas exchange. Consecutively, the solution was stirred at 100 rpm. For comparison purposes with the steel-making slag, iron sulfate (FeSO$_4$) (Sigma-Aldrich Japan) and iron powder (Sigma-Aldrich Japan) with 325 $\mu$m average diameter were also tested following the same procedure. These elements were added to artificial seawater to make 30.2 mg-Fe similar to the iron dosage of steel-making slag. The control without any iron sources was also set up. All the experiments were conducted in triplicates.

2.2.2. Sampling and Chemical Analyses

All glassware and low density polyethylene bottles (LDPE, Nalgene) for sample stock were soaked in 12 M HCl bath for 12 h and rinsed thoroughly with ultra-pure water before use. Samples were collected from the flasks 1 hour after the addition of the respective Fe sources, and thereafter every other day with a syringe, and pH was mea-

\begin{table}[h]
\centering
\caption{Chemical composition of the slag used in this study.}
\begin{tabular}{ll}
\hline
Substances & (\%)
\hline
CaO & 41.47 \\
SiO$_2$ & 23.53 \\
Total Fe & 15.06 \\
MgO & 6.20 \\
MnO & 3.74 \\
P$_2$O$_5$ & 2.33 \\
Al$_2$O$_3$ & 1.62 \\
TiO$_2$ & 1.04 \\
Cr$_2$O$_3$ & 0.62 \\
S & 0.05 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Chemical composition of artificial seawater.}
\begin{tabular}{ll}
\hline
Substances & (g)
\hline
Distilled water & 1 000 ml \\
NaCl & 24 g \\
KCl & 0.6 g \\
MgCl$_2$·6H$_2$O & 3.0 g \\
MgSO$_4$·7H$_2$O & 5.0 g \\
CaCl$_2$·2H$_2$O & 0.4 g \\
KBr & 1.19 mg \\
SrCl$_2$·2H$_2$O & 27.0 mg \\
H$_2$BO$_3$ & 6.0 mg \\
NaF & 0.42 mg \\
KI & 33.0 mg \\
NaHCO$_3$ & 0.2 mg \\
\hline
\end{tabular}
\end{table}
The growth experiments was determined by fluorometry. Furthermore, the fraction was filtered through a 0.22 μm membrane filter (Millex HV25, Millipore) and analytical grade HCl (Wako Pure Chemical) was added to the filtrate to make a 0.01 M final concentration in order to maintain its pH at 2–3 until analyses. Some of the fraction passing through the 0.22 μm membrane filter was further filtered through a 50 KDa ultrafiltration filter (Ultrafilter Q500, Advantec) and its pH was adjusted to 2–3 by analytical grade HCl as above. These samples were kept at 5°C in the dark until further analysis. In this study < 50 KDa fraction and > 50 KDa to < 0.22 μm fraction were defined as dissolved iron and colloidal iron, respectively.

Total iron and Fe(II) in these samples were analyzed by chemi-luminescence method coupled with chelating resin concentration column. Concentration of Fe(III) was calculated by subtracting the concentration of Fe(II) from the total iron. Colloidal fraction was determined with the same method described above.

2.2.3. Growth Experiments of Skeletonema costatum

Artificial seawater was prepared following the dissolution test described in previous section. After preparing the artificial seawater, the cultured strain Skeletonema costatum, which is one of the common diatom species in Japanese coastal waters, was added to the artificial seawater to make a final concentration of 1 × 10⁶ cells L⁻¹. Then, 200 mg of UV sterilized steel-making slag was added to the seawater and the flask was capped with a sterilized silicon cap to allow gas exchange. S. costatum was cultured under conditions of 15°C and 200 μmol photons m⁻² s⁻¹ (12L:12D) by stirring at 100 rpm. The other iron sources such as FeSO₄ (Sigma-Aldrich Japan) and iron powder (Sigma-Aldrich Japan) with 325 μm average diameter were also tested following the same procedure to compare them with that of the steel-making slag. Those iron sources were added to artificial seawater to make 30.2 mg-Fe similar to the iron dosage of steel-making slag. The control without any iron sources was also set up. Sampling and chemical analyses were carried out as described in the dissolution test. Furthermore, 5 mL of sample was filtered through a GF/F filter (Whatman), and the filter together with the residue on it was soaked in 10 mL of N,N-dimethylformamide to extract chlorophyll a (Chl a). The concentration of Chl a in the extracts was determined by fluorometry. The growth experiments of S. costatum were conducted in triplicates.

3. Results and Discussion

3.1. Dissolution Test

The pH values of the solution with iron powder and in the control flasks were almost stable (Fig. 1). On the other hand, the pH of the solution with slag increased to 8.3, while that with FeSO₄ decreased to pH 7.0 just after the addition of FeSO₄.

The increase in pH with the addition of the slag is caused by the dissolution of CaO contained in the slag as shown in Eq. (1). However, seawater exhibits a carbonate buffer action as shown in Eq. (2). Therefore, the increase in pH is maintained within the range not affecting the growth of phytoplankton.

\[
\text{CaO + H}_2\text{O} \leftrightarrow \text{Ca(OH)}_2 \leftrightarrow \text{Ca}^{2+} + 2\text{OH}^- \quad (1)
\]

\[
\text{CO}_2 + \text{H}_2\text{O} \Leftrightarrow \text{H}_2\text{CO}_3 \Leftrightarrow \text{H}^+ + \text{HCO}_3^- \Leftrightarrow 2\text{H}^+ + \text{CO}_3^{2-} \quad (2)
\]

The decrease in pH seen with the addition of FeSO₄ is caused by the hydrolysis of iron. As described later, FeSO₄ dissolved rapidly in the artificial seawater and the concentration of dissolved iron increased dramatically when compared to the other flasks. During the hydrolytic process of dissolved iron into iron hydroxide, protons were simultaneously released (Eq. (3)). Since seawater has a carbonate buffer reaction as mentioned above, the pH returned to normal levels thereafter.

\[
\text{Fe}^{3+} + 3\text{H}_2\text{O} \rightarrow \text{Fe(OH)}_3 + 3\text{H}^+ \quad .............. (3)
\]

The dissolved Fe(II) concentration in the FeSO₄ flask increased to 0.33 μM on Day 1, but decreased gradually afterwards (Fig. 2(a)). The Fe(II) concentration in the slag flask increased gradually to 3.0 nM on Day 9, though it would not be identified in the figure. However, Fe(II) was not detected in the other flasks. The dissolved Fe(III) concentration in the FeSO₄ flask increased to 12.2 nM, just after the addition of FeSO₄ (Fig. 2(b)). However, it decreased rapidly and was not detected after Day 7. These levels of dissolved Fe(III) in the other flasks were below the detection limit (0.2 nm) of the chemi-luminescence method coupled with chelating resin concentration column used in the present study applied for Fe(II) and total dissolved iron (Fig. 2(b)).

The colloidal Fe(II) concentration in the FeSO₄ flask increased to 0.85 μM, following the addition of FeSO₄, and decreased rapidly below the detection limit (Fig. 3(a)). The Fe(II) concentrations in the other flasks were at the basal level. The colloidal Fe(III) concentration in the FeSO₄ flask increased to 3.2 μM just after FeSO₄ addition. However, it decreased rapidly and was not detected after Day 1 (Fig. 3(b)). The colloidal Fe(III) concentration in the slag flask increased to 1.1 μM on Day 1 and decreased gradually after
Colloidal Fe(III) in the iron powder and the control flasks was not detected, except for Day 9 in the iron powder flask (Fig. 3(b)).

Even though iron in FeSO₄ is divalent, the Fe(III) concentration in the FeSO₄ flask also increased dramatically (Fig. 2(b)). Since the oxidation rate of Fe(II) in seawater is very fast,28,29) Fe(II) from iron oxide was quickly oxidized to Fe(III). The half-time from Fe(II) to Fe(III) is 2.8 min at pH 8, 20°C.29) Furthermore, the dissolved iron in the FeSO₄ flask quickly changed into iron hydroxide/oxyhydroxide, successively turning into colloidal iron (Fig. 3) because of the very low solubility of Fe(III) in seawater, then decreased significantly within one week.30) Afterwards, colloidal iron may grow by aggregation into large amorphous iron hydroxide or particulate iron with sizes bigger than 0.22 μm. Therefore, the colloidal iron concentration decreased rapidly within a day.

In contrast, a slight increase in dissolved Fe(II) was observed in the slag flask as mentioned above (Fig. 2(a)). The dissolution rate of slag was much slower than that of FeSO₄, which implies that the concentration of dissolved iron did not exceed its solubility maximum in the slag flask, compared to that of the FeSO₄ flask. Therefore, the hydrolysis and aggregation rate in the slag flask are also slower than those of the FeSO₄ flask. Thus, only the slag did dissolve Fe(II) in moderate concentrations. Although the available iron species for phytoplankton is not clear so far,31) the slag used in this study is suitable for the growth of phytoplankton providing useful dissolved iron, probably Fe(II), in comparison to FeSO₄ and iron powder.

Changes in DIP and DSi concentrations are shown in Fig. 4. The dissolution of DIP and DSi, which is essential for the growth of phytoplankton, was only observed in the slag flask. As shown in Table 1, the slag contains phosphate and silicate which come from the purification process of pig iron.

According to the extended Redfield ratio, phytoplankton cells uptake P, silicon (Si), iron (Fe) at a ratio of 1:15:0.0075.32,33) Therefore, the molar ratios of Si/P and P/Fe become 15 and 1000, respectively. The changes in the Si/P and P/Fe ratios in the slag flask occurring over time are calculated using DIP, DSi and dissolved Fe(II)+Fe(III) concentrations. As shown in Fig. 5, the molar ratios of Si/P and P/Fe are around the Redfield ratio. This implies that the slag used in this study could be very effective in supporting the growth of phytoplankton.

3.1.1. Growth Experiments of Skeletonema costatum

The change of Chl a concentration in the culture experiment is shown in Fig. 6. A significant increase in Chl a was observed only in the slag flask with the concentration of 14 μg L⁻¹ on 7th day and finally 280 μg L⁻¹ at the end.
of the experiment. This kind of delay in the growth of phytoplankton is due to “lag” in growth when they transplanted to new culture media which is of common phenomenon in their physiology of microalgae, and in addition, it could be the effect of increase in pH. On the other hand, Chl \( \text{a} \) concentrations of the FeSO\(_4\) flask, iron powder flask, and control flask were 0.24, 0.36 and 2.1 \( \mu \text{g L}^{-1} \), respectively, at the end of experiment. The high value in the slag flask could be attributed to the essential nutrients released from the slag such as phosphate, silicate and iron, which promoted the growth of \textit{Skeletonema costatum} (Fig. 4).

A recent study on the culture and biofuel production efficiency of \textit{S. costatum} has demonstrated that the addition of nitrate, phosphate and silicate to the culture medium increased the growth efficiency of this diatom species.\(^{15} \) The biomass of \textit{S. costatum} was reported to have increased from a minimum of 0.1125 mg/10 ml on the first day to a maximum on 0.6198 mg/10 ml on the 12th day of the growth experiment.\(^{15} \) Furthermore, since \textit{S. costatum} has a rich total lipid content of 21.6%, the biodiesel yield of this diatom in terms of weight (biodiesel g/g dry-weight of biomass) relative to the algal oil present in the biomass is the highest (0.87), when compared to those of other microalgal species like \textit{Chlorella marina} (0.70), and oil seed crops like \textit{Jatropha} (0.62), coconut (0.57), groundnut (0.51), white soy bean (0.38), castor (0.33) and red soy bean (0.09). Therefore, the high growth efficiency and rich oil composition of \textit{S. costatum} make this diatom a promising target species for biofuel production and carbon dioxide (CO\(_2\)) mitigation.

Our work has proved that the dephosphorized slag released iron ions as dissolved and colloidal Fe(III) moderately into the surrounding water, and the concentration of dissolved iron was maintained. These characteristics were explicitly different from those of the other iron sources, such as iron powder and FeSO\(_4\). Particularly, colloid formation by FeSO\(_4\) would not be as efficient for the growth of phytoplankton. In addition, DIP and DSi were also released from the dephosphorized slag, and the ratio, including released iron, was close to the extended Redfield ratio of P, Si, and Fe as 1:15:0.0075, which is the average ratio of the major nutrients constituting phytoplankton cells. In addition, the experiment conducted with the common marine diatom \textit{S. costatum} showed that the slag enhanced the growth of this species. However, we have to find out any nitrogen source

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**Fig. 4.** Change in the concentration of dissolved inorganic phosphorus (DIP) and dissolved silicon (DSi) overtime in each flask.

**Fig. 5.** Change in the molar ratio of P:Si and P:Fe over time in the slag flask.

**Fig. 6.** Change in the concentration of chlorophyll \( \text{a} \) (Chl \( \text{a} \)) overtime at each flask.
with less cost for mass culture of algae. For example, treated sewage water is suggested. Therefore, this work constitutes a pioneer research focusing on steel-making slag as source of P, Si and Fe except N source for phytoplankton growth.

The present study is the first step in utilizing steel-making slag for the mass culture of marine phytoplankton. We selected a marine phytoplanktonic species for our study, assuming the application of the present results in the areas around Fukushima and elsewhere where the land was inundated with saline water as a result of the tsunami of 11th March, 2011. Not only utilizing patty fields directly for culture pond of marine algae if the land owner abandon to use them for rice production, but also it is comparatively easy in terms of air-borne contamination because freshwater microorganisms generally cannot grow in seawater. Furthermore, in culture of marine phytoplankton species along the shoreline, we can reduce the cost for freshwater supply. Although steel-making slag has already been utilized as fertilizer for rice production in Japan, further studies on steel-making slag are also required because characteristics of slag are different depending on the processes of companies.

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