Chapter 2

Quality Assessment of Microalgae Exposed to Trace Metals Using Flow Cytometry

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Additional information is available at the end of the chapter

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Abstract

Seaweed has long been an important kitchen ingredient and a functional food material. Microalgae have attracted the same attention as seaweed from food, pharmaceutical and cosmetic companies because several algae contain unique functional materials. Industry application of algae requires the selection of useful algal species, evaluation of their features and monitoring of their quality in culture. Taking *Chlorella* for example, this chapter presents a method using flow cytometry (FCM) to assess not only the number of algae but also algal quality. First, *Chlorella* was cultured in media containing eluate from steel slag as an experimental factor and trace metals. After the treatment of algae with eluate, the number and physiological features of algae were evaluated, respectively, using hemocytometry and FCM. Results show that eluate from slag induced neither lethality nor growth inhibition. Coupled with hemocytometry, FCM was used to estimate vigorous and aberrant algal status. Consequently, the eluate did not give rise to algae stresses. Interestingly, the addition of slag eluate increased the amounts of the carbonate species. The increase in the carbonate species actually triggered the potential increase in aqueous CO_2 for photosynthesis, eventually inducing algal proliferation. These analyses can support evaluation of algal features and maintenance of their quality for industry application.

Keywords: food science, *Chlorella*, Chlorophyll, flow cytometry, fluorescence spectroscopy, trace elements, steel slag

1. Introduction

Aqueous photosynthetic organisms such as algae are the foundation of aquatic ecosystems. The quantities of aqueous photosynthetic organisms as producers in the aqueous ecosystem

support yields of both fish and shellfish. In addition to its role as a producer in aqueous ecosystems, seaweed has long been an important kitchen ingredient as a functional food material, providing functional nutrients such as carotenoids and anti-oxidative fucoxanthin. A biorefinery that can take advantage of biofunctions presents an alternative concept to that of conventional refineries that manufacture materials using fossil fuels. Especially, autotrophic algal biorefineries present great advantages over conventional microbial biorefineries such as those using fermentation. Some microalgae species such as *Chlorella* spp., *Dunaliella salina* and *Haematococcus pluvialis* are appreciated respectively as sources of β-1, 3-glucan, β-carotene and astaxanthin [1]. They have attracted attention equal to that devoted to seaweed products from several pharmaceutical, vitamin supplement, cosmetic and food companies [1, 2] because these algae have functional materials that are rare among land plants. Moreover, other microalgae have attracted attention for use as biofuel materials [2, 3] and bioremediation materials for environmental biodegradation [4, 5].

The industrial application of algae demands the selection of useful algal *sp.,* the evaluation of algal features and the assessment of their qualities in culture. Open pond culture systems, rather than closed systems, are the main type of culture system for the commercial scale culture of microalgae because of their relative low cost [6]. As commonly known, human activities have major impacts on the global and regional cycles of most of the trace elements including toxic heavy metals [7]. Atmospheric transport and deposition are potentially important processes for delivering a wide range of anthropogenic contaminations to aquatic environments [7, 8]. Microalgae are very sensitive to changes in their environment [9]. Their overall metabolisms are greatly affected by even trace amounts of various organic and inorganic pollutants including heavy metals [9]. Such fear factors might pose a threat to open culture systems of algae. Therefore, it is especially important to routinely control and manage algal qualities in culture.

Taking green algae *Chlorella* spp., for example, this chapter presents a method to assess algal quality using flow cytometry (FCM). *Chlorella* was cultured in media containing eluate from steel slag as an experimental factor and trace metals. After treatment of algae with eluate, the number and physiological features of algae were evaluated respectively using hemocytometry and FCM. These analyses are expected to contribute to the evaluation of algal features and to the maintenance of their quality for industry applications.

2. Algal characteristics using FCM

Over the last few decades, FCM has become widely used as a powerful and valuable tool for studies of cell biology, microbiology, protein engineering and healthcare. Several functions of FCM include several procedures such as cell counting, biomarker detection and cell sorting through assessment of cell optical information. **Figure 1** presents an outline of a flow cytometric instrument used for this study. This flow cytometer, which detects several optical properties, is equipped with a green laser operating at 532 nm. Forward scatter (FSC) signals were collected to ascertain the cell size. Red fluorescence is detected in the red fluorescence

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channel through a 680/30 nm band pass filter. Simultaneously, a yellow fluorescence channel through a 576/28 nm band pass filter is used [10–13]. Each fluorescence is converted into an electrical pulse. The electrical intensity is then quantified for each level of fluorescence intensity.

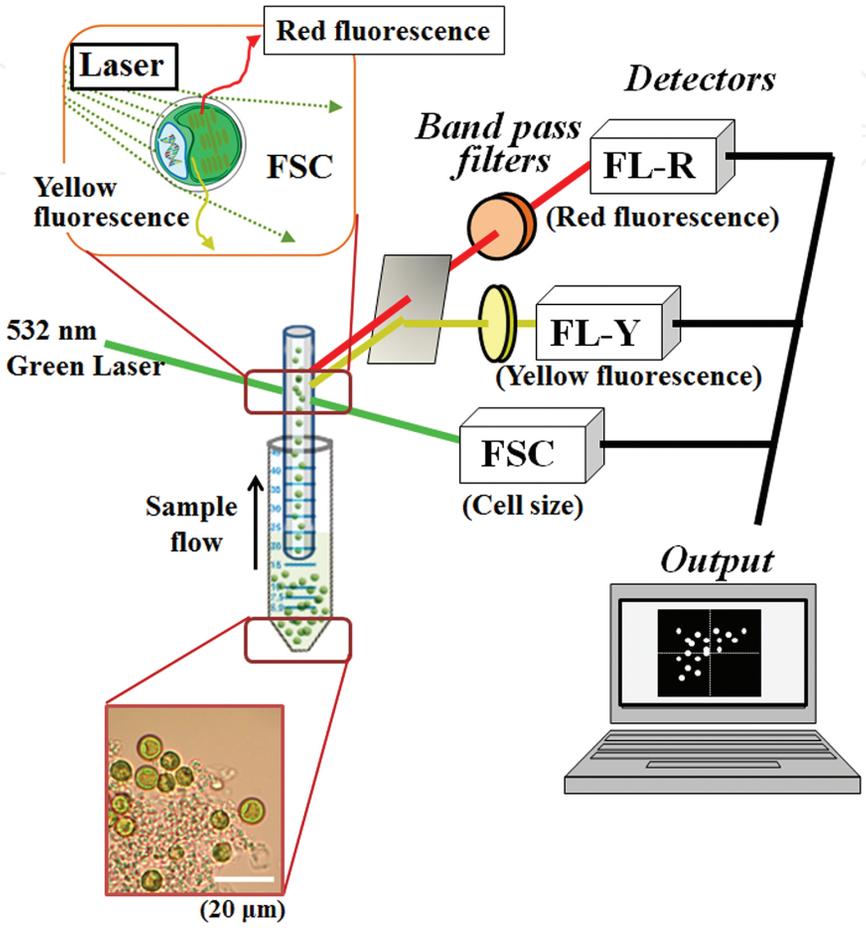

Figure 1. Overview of the flow cytometric system used for this study. Algae that had passed through a capillary were analysed. In addition to the red and yellow fluorescence derived from algae, FSC signals of algae were collected simultaneously as shown.

When heterotrophic cells, such as animal cells, are targeted for FCM measurement, fluorescence-labelling antibodies against certain biomolecules are used to detect and quantify the biomolecules. When using phototrophic cells, such as phytoplankton and plant cells, a photosynthetic pigment, chlorophyll, can also function as a biomarker similar to a fluorescence labelling antibody. When exposed to appropriate excitation light, chlorophyll in each cell irradiates red fluorescence (**Figure 2A** and **B**) [12]. **Figure 2C** depicts emission spectra of *Chlorella*-like algae [14, 16]. The wavelength of the maximal fluorescence of algal chlorophyll is approximately 680 nm (green curve in **Figure 2C**). Consequently, chlorophyll fluorescence is mainly detectable using the red fluorescence channel of the instrument used for this study. The cell size and chlorophyll content of algae are correlated strongly with the algal cell cycle [14–16].

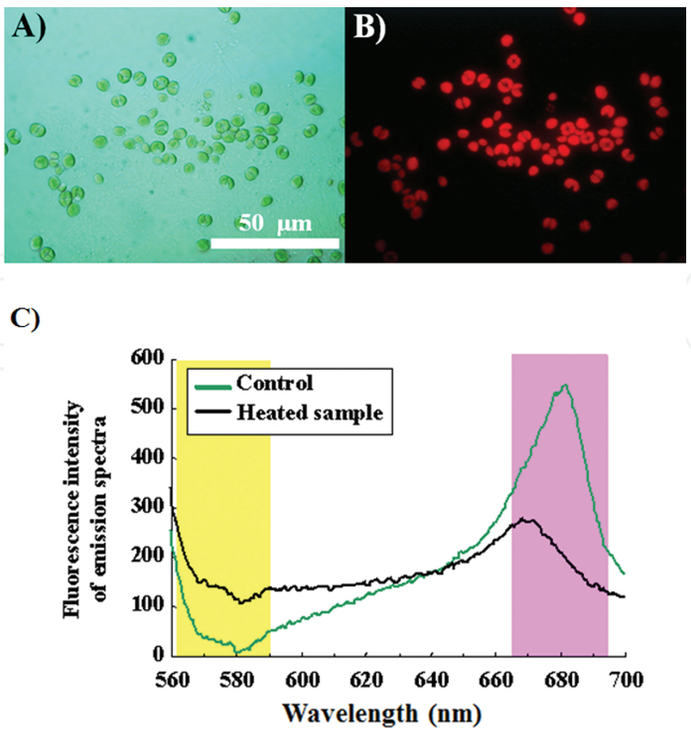

Figure 2. Fluorescence characteristics of algae and microphotographs of *Chlorella*-like algae isolated from protozoa *Paramecium bursaria*. Algal images obtained using bright field (A) and fluorescence microscopy (B) were referred from the literature [12]. Panel C presents fluorescence characteristics of algae obtained using fluorescence spectroscopy referred from the literature [10]. Emission spectra of algae are shown with (black line, heated algae) or without (green line, control algae). Yellow and pink areas, respectively, show detection ranges of yellow and red fluorescence channels used for FCM in this study.

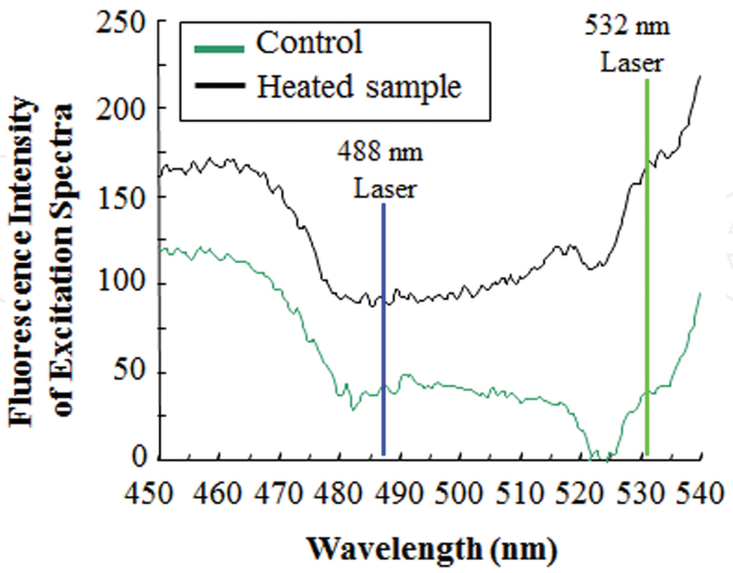

Figure 3. Excitation spectra of *Chlorella*-like algae with or without heat treatment referred from the literature [10]. The fluorescence intensities at 575 nm were measured to produce excitation spectra. Two vertical lines signifying 488 nm (blue) and 532 nm (green) are shown in the graph.

Chlorophyll is sensitive to physiological factors such as heat and acid. These physical factors eventually cause inactive chlorophyll because of degradation [11–13]. In fact, a previous study using *Chlorella*-like algae [10] demonstrated that algae without stress irradiated only red fluorescence derived from chlorophyll (green curve in **Figure 2C**). In contrast, dead algae, subjected to extraneous stress, tended to have less red fluorescence and more yellow fluorescence because of the biodegradation of chlorophyll (black curve in **Figure 2C**). Moreover, this instrument presents benefits for evaluation of algal status because the excitation efficiency of the green laser at 532 nm in the yellow fluorescence is higher than that of a conventionally used blue laser at 488 nm (**Figure 3**) [10]. It suggests that the red and yellow fluorescence intensities are regarded respectively as indices of vigorous algae and of variant algae when the green laser irradiates algae moving through the capillary of this flow cytometer [11, 13].

3. Features of steel slag used for this study

Iron and steel slags from blast furnace slag and steel making slag including converter slag and electric arc furnace (EAF) slag are produced as steel industrial by-products. All blast furnace slag can be recycled completely for the use of steel making slag base and cement as soil aggregate [17, 18], although several volumes of steel making slag, particularly EAF slag, ultimately end up in landfill sites [19]. New applications of slag, such as depurative and sand capping materials in aquatic environments [20] have been regarded both as decreasing the amounts of discarded slag and as reducing high costs of discarding slag. Several environmental pollution laws, however, have restricted slag use in aquatic environments because steel slag contains environmentally hazardous substances. The toxicity of eluate from EAF slag for aquatic organisms remains poorly understood [11, 13, 21], but the physiochemical properties and effects of converter slag on organisms have been documented often [22–27].

This study specifically examined stainless steel slag (designated as slag A) and common steel slag (slag B), exhausted respectively from oxidation processes of stainless and common steelmaking in EAF processes [11, 13]. **Table 1** presents compositions of EAF slags used for this study [11, 13, 21, 28, 29]. In brief, slag A contains more SiO_2, CaO, and Cr_2O_3 than slag B does, whereas slag A contains less FeO than slag B. All Fe and Cr compounds are described respectively as FeO or Cr_2O_3 because it is generally difficult to distinguish FeO and Cr_2O_3 formed form Fe and Cr in a suspended metal solution after alkali fusion of stainless steel slag [11, 13].

	FeO	**SiO_2**	**CaO**	**Al_2O_3**	**MgO**	**MnO**	**Cr_2O_3**	**ZnO**	**NiO**	**CuO**
Slag A	0.74	44.1	33	5.39	7.68	4.09	3.29	0.01	0.06	0.024
Slag B	35.1	19.2	20.8	15.2	4.1	5.1	0.43	0.071	0.028	0.025

Table 1. Chemical compositions of EAF steel slags used for this study (mass %).

4. Research methods

The author used *Chlorella* as the model organism representing algae in this study. Several methods used to examine algal behaviours have been established using *Chlorella* spp. The author used *Chlorella kessleri* (C-531 strain) which was obtained from the Institute of Applied Microbiology (IAM) culture collection at The University of Tokyo. The scientific name of *C. kessleri* was recently changed to *Parachlorella kessleri* because the taxonomy of *Chlorella* has been re-validated using multidisciplinary approaches based on combining classical and modern methods including molecular phylogeny and bioinformatics [30]. Before experiments, algae on the CA agar plates [31] were scratched with an inoculating needle and were suspended in CA liquid medium.

Steel slag was subjected to a leaching test based on JIS K0058-1: 2005 (Method for chemicals in slags Part 1: Leaching test) to elute metal components of slag with HCl [11, 13, 21, 28, 29, 32]. After elution, the solution was filtrated with a 0.45 μm pore filter to eliminate slag particles as described in previous reports. The filtrated eluate from the slag (designated respectively as eluate A and eluate B) was used for bioassay with *Chlorella* as a test solution including trace metals.

To assess the eluate effects on algal growth, *Chlorella* was cultured in CA medium containing an eluate from steel slag as an experimental factor and sources of trace metals [11, 13]. Compared with general culture media for algae, slag eluates used for this study contained insufficient nutrients for algal growth. To supplement nutrients for algal growth, the following assessments of algal growth were conducted with CA liquid medium at pH 7.2 [11, 13]. Nutrient amounts of CA medium containing eluates were the same as those of CA medium alone, but the concentrations of chemicals derived from each eluate differed from those of CA medium without eluate. Here, CA medium without eluate was designated as "control".

Algae (initial density of 1.0×10^4 cells/ml adjusted using hemocytometry) were cultured with CA medium containing eluate from each slag for 1 week in a plastic tube under an LD cycle (12 h light/12 h dark) at approximately 1100 lux of natural white fluorescent light and 23 ± 2°C as described in previous reports [11, 13]. After treatment of algae with eluate, the number and physiological features of algae were quantified respectively using hemocytometry and FCM. The algal proliferation ratio (average ± standard error) was expressed as a proportion of the number of algae treated with eluate to that of control without eluate [11, 13].

To investigate algal status using FCM, the algal status was analysed and estimated based on the corresponding fluorescence. In brief, the stress of each alga is portrayed as a two-dimensional graph (2D map) of red fluorescence intensity (665–695 nm) as the index of vigorous algae and yellow fluorescence intensity (562–590 nm) as the index of variant algae [11, 13]. To facilitate comparison of vigorous algae with stressed and dying algae, a reference standard of algae subjected to stress was prepared by treatment of algae with heat for 5 min at 100°C (designated as heated algae) [11, 13]. For FCM analyses, FSC signals detected only in the culture medium were removed as technical noise from FCM measurements as described in previous reports [11, 13]. The remaining signals were re-analysed as algal signals.

Aquatic CO_2 (CO_2(aq)) concentrations are related directly with photosynthesis and algal proliferation. CO_2(aq), HCO_3^- and CO_3^{2-} are present as the carbonate species in solution, as presented in Eqs. (1)–(3) [11, 13].

$$CO_2(\text{gas}) \leftrightarrow CO_2(\text{aq}) \tag{1}$$

$$H_2O + CO_2(\text{aq}) \leftrightarrow H_2CO_3(\text{aq}) \leftrightarrow HCO_3^- + H^+ \tag{2}$$

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \tag{3}$$

For aqueous photosynthetic organisms, CO_2(aq) of these carbonate species is particularly necessary to support photosynthesis. We ascertained the concentration of CO_2(aq) in slag eluate using potentiometry with a diaphragm-type electrode to measure the CO_2(aq) concentrations [11, 13]. Both HCO_3^- and CO_3^{2-} can be estimated as CO_2(aq) in acidic conditions (≤ pH 4.0) as portrayed in **Figure 4** resulting from the following Henderson–Hasselbalch Eqs. (4) and (5) [11, 13].

$$pH = pK_1 + \log([HCO_3^-]/[CO_2(\text{aq})]) \tag{4}$$

$$pH = pK_2 + \log([CO_3^{2-}]/[HCO_3^-]) \tag{5}$$

The respective pK values of pK_1 = 6.35 and pK_2 = 10.33 [33] were used for this study. The carbonate species aside from CO_2(aq) were converted into CO_2(aq) by adding a pH-adjustable solution. Then they were estimated as the amounts of total carbonate species. Each concentration of CO_2(aq), HCO_3^-, and CO_3^{2-} was calculated from the amounts of total carbonate species and pH values using Eqs. (4) and (5) above. Here, the [H_2CO_3(aq)] given by Eq. (2) was expressed as [CO_2(aq)] in Eq. (4) because it was difficult to distinguish CO_2(aq) from H_2CO_3(aq) in solution, as described in previous reports [11, 13]. The result was expressed as the concentration of CO_2(aq) (average ± standard error) under each condition [11, 13].

In general, the amounts of Ca^{2+} and Mg^{2+} are related to the water hardness and are highly reactive with carbonate species. To examine whether these elements contribute to the concentration of CO_2(aq) in solution, the amounts of Ca^{2+} and Mg^{2+} were measured before and after treatment of algae with CA medium containing slag eluate [11, 13]. After treatment of algae with CA medium containing eluate, the culture tube including the algae was centrifuged. The supernatant, which no longer included algae, was collected and subjected to elemental analysis. Several organic compounds, such as biomolecules reportedly interfere with these measurements [34]. Therefore, this study applied colorimetric determination using specific chelate reagents to elucidate the amounts of Ca^{2+} and Mg^{2+} in the culture supernatant. In

practice, the chlorophosphonazo-III method [35] and the xylidyl blue-I method [36] were used for the evaluation of the concentration of Ca^{2+} and that of Mg^{2+}. Moreover, elemental concentrations before treatment of algae with eluate were compared statistically with those after treatment using *t*-tests.

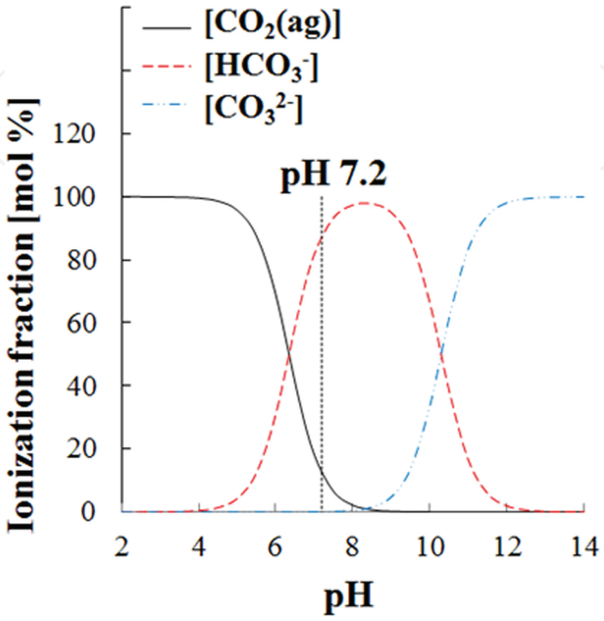

Figure 4. Concentrations of CO_2(aq), HCO_3^-, and CO_3^{2-} for each pH [mol%] referred from the literature [11, 13].

5. Results and discussion

Before evaluating the effects of slag eluates on algae, the concentrations of elements in each slag eluate were analysed and discussed (**Table 2**). In addition to the results of leaching tests for slag, the environmental quality standards (EQSs) for soil pollution and for marine and water pollution are shown as reference values in **Table 2** [13]. In brief, concentrations of Ca, Mg and Si eluted from the slag samples were high because these slags contained large amounts of those materials (**Table 1**). In contrast to the elements above, the eluted concentrations of Al and Fe were quite low in spite of their high concentrations in the slag particles. An earlier report [32] explained this contradictory phenomenon as a difference of these elements in terms of solubility. Leaching tests revealed that concentrations of components eluted from two slags used for this study were almost all lower than the respective EQSs, except for selenium (Se) in eluate from the slag A (eluate A), as described in earlier reports [11–13].

This study examined the effects of slag eluates as an experimentally stress factor on algal growth, particularly that of *Chlorella* spp. [11–13]. **Figure 5** shows the relation between the *Chlorella* proliferation ratio and concentrations of the slag eluate in the test solution. Here, all nutrient amounts derived from CA medium, other than elements derived from each slag eluate, were constant with each experimental condition. A detailed account of the results

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showed that the number of algae increased according to the concentration of each eluate up to 30 vol%. Subsequent comparison of the algal proliferation ratios in eluate A and eluate B showed that these ratios were almost equal at concentrations lower than 50 vol% of the respective eluates. However, the 70 vol% of eluate B showed a slightly more algal proliferation than that of eluate A, as described in previous reports [11–13]. After explaining the results from FCM analysis, we subsequently discussed the difference in the algal proliferation ratio between eluate A and eluate B.

Origin of slag		**Eluate of EAF stainless steel oxidation slag (Slag A)**	**Eluate of EAF normal steel oxidation slag (Slag B)**	**Environmental quality standards**				
				Soil pollution	**Marine pollution**	**Water pollutant**	**Effluent standard**	**Drinking water standard**
Regulated substances	Total As	ND[1] (RDL[2]: 0.001)	ND[3]	0.01	0.1	0.01	0.1	0.01
	Total B	0.16	0.28[3]	1		1[4]	10[4], 230[5]	1
	Total Be	ND (RDL: 0.0005)	ND[3]		2.5			
	Total Cd	ND (RDL: 0.0001)	ND[3]	0.01	0.1	0.01	0.03[6]	0.003
	Chromium (VI)	ND (RDL: 0.005)	ND[3]	0.05	0.5	0.05	0.5	0.05
	Total Cu	0.003	ND	0.001	3		3	1
	Total Pb	ND (RDL: 0.0005)	ND[3]	0.01	0.1	0.01	0.1	0.01
	Hg	ND (RDL: 0.0001)	ND[3]	0.0005	0.005	0.0005	0.005	0.0005
	Total Ni	0.001	ND	0.001	1.2			0.02
	Total Se	0.012	0.003[3]	0.01	0.1	0.01	0.1	0.01
	Total V	ND (RDL: 0.001)	0.01[3]		1.5			
	Total Zn	0.099	0.014[3]		2	0.03[7], 0.02[9], 0.01[9]	2	1
	F^-	ND (RDL: 0.1)	0.5[3]	0.8	15	0.8[4]	8[4], 15[5]	0.8

Origin of slag		Eluate of EAF stainless steel oxidation slag (Slag A)	Eluate of EAF normal steel oxidation slag (Slag B)	Environmental quality standards				
				Soil pollution	Marine pollution	Water pollutant	Effluent standard	Drinking water standard
Substances out of regulation	Total Al	ND	1.8					
	Total Ca	9.3	10.1					300[10]
	Total Fe	ND	0.23				10	0.3
	Total Mg	0.9	1.1					300[10]
	Total Mn	0.028	ND				10	0.05
	Total Si	1.8	1.9					
	Total N	0.4	0.362[3]			0.1–1[11] 0.2–1[8]	100	0.04[12], 10[13]
	Total P	ND (RDL: 0.1)	ND[3]			0.005–1[11] 0.02–0.09[8]	16	

[1]Not detected.

[2]Reportable detection limit.

[3]These data from a previous study reported by Takahashi et al. [17].

[4]Standard value is not applied to coastal waters.

[5]Standard value is applied to coastal waters.

[6]The Cd value has changed from 0.1 to 0.03 mg/L since December 2014.

[7]Habitable river or lake for aquatic life.

[8]Habitable coastal water for aquatic life.

[9]Habitable coastal water that requires conservation in particular for nidus and nursery ground.

[10]Total concentrations of both calcium and magnesium are limited for water hardness.

[11]Habitable lake for aquatic life.

[12]Total N contents derived from nitrite nitrogen.

[13]Total N contents derived from both nitrite nitrogen and nitrate nitrogen.

Table 2. Environmental quality standards regarding pollutions and others for effluent and drinking water, and concentrations of elements of each eluate (mg/L) quoted with permission from Ref. [9].

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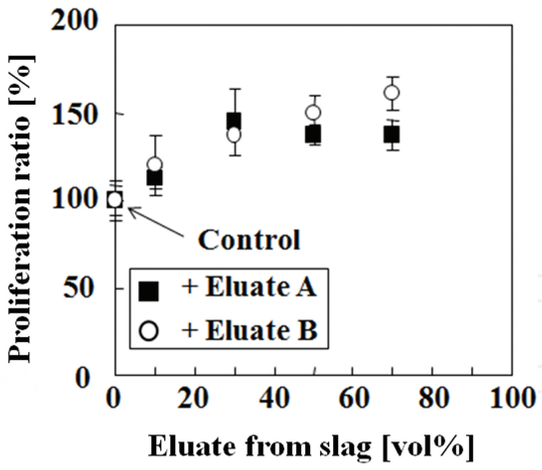

Figure 5. Effects of respective eluates on algal growth referred from the literature [11, 13].

In addition to algal population estimation using hemocytometry, estimation of the cellular status of algae using FCM was conducted. The results are presented two-dimensionally in **Figure 6** [11–13]. Here, each single dot on the 2D map represents optical information of a single alga. To compare the status of vigorous algae with that of stressed and dying algae, algae treated with heat (heated sample) were prepared as a reference standard of algae subjected to stress [11–13]. Results show that the 2D map of the red versus the yellow fluorescence intensity for control algae differs clearly from that for the heated algae (**Figure 6**). The 2D map of the red versus yellow fluorescence intensity for control algae showed respectively 10^2–10^3 on the red channel and 10^1–10^2 on the yellow, whereas that for the heated algae did 10^1–10^2 on the red channel and 10^1–10^3 on the yellow. It is particularly interesting that the dot distribution of algae treated with each slag eluate closely resembled that of control, although that with each eluate shifted slightly upward relative to that of control algae [11–13].

Quantitative analysis of algal distribution patterns (**Table 3**) was conducted along with qualitative analysis of those patterns (**Figure 6**). Each graph in **Figure 6** is divisible into four subareas (regions I–IV) based on algal viability [11–13]: region I represents an area for vigorous algae; region II includes dead and variant algae such as heated algae; region III includes algae with low red fluorescence intensity; and region IV includes data from which algae are virtually absent. Algal distribution patterning revealed clear differences between the algal distribution in control samples and those in heated algae. The signals of algae treated with eluate were also distributed almost entirely to region I. The ratio was 96.81 ± 2.60% in control, 98.15 ± 0.31% in eluate A, and 98.13 ± 0.24% in eluate B. Ratio analysis shows that the percentages of algae treated with slag eluates were slightly higher than those of control algae. Components dissolved from slags did not apparently give rise to algae stress because the quantities of algae in media containing the respective eluates were equal to or greater than those in media with no eluate (**Figure 5**). The tested slags contain metals such as copper, zinc and aluminium (**Table 2**). Aluminium, which is also not contained in the CA medium, has been particularly reported as inhibiting plant growth [37, 38]. Although the culture medium containing aluminium and other metal elements was predicted to affect algal growth and status, they

caused no effect on algal growth directly. The data demonstrated that components eluted from slag showed no marked toxicity to algae. This assessment system using FCM, which estimates chlorophyll fluorescence of photosynthetic pigments, might be applicable to other algae, other phytoplankton and aquatic plants with chlorophyll, although this report presents data only for *Chlorella* spp. as a model organism. This technique can contribute to evaluation of algal features and monitoring their qualities in culture for industry application of algae.

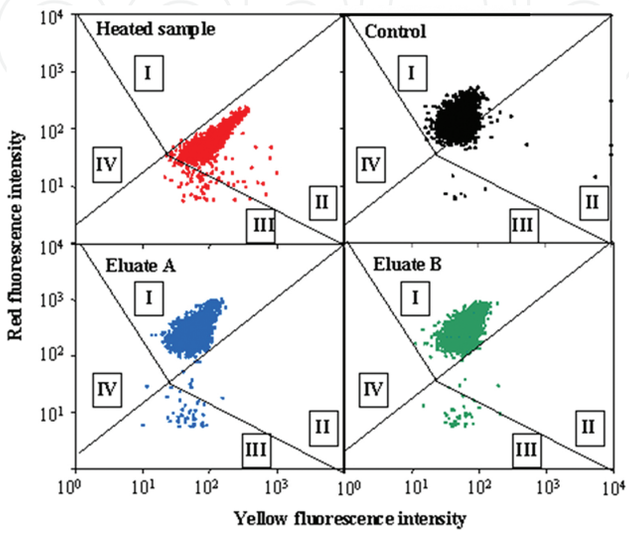

Figure 6. Distribution of *Chlorella* using FCM referred from the literature [11–13]. The red fluorescence intensity of algae is shown versus the yellow fluorescence intensity. The heated sample is the heat treatment sample of algae. Eluate A and eluate B denote solutions with respective concentrations of eluate A and eluate B of 50 vol%.

	I	II	III	IV
Control	96.81 ± 2.60	2.40 ± 2.91	0.73 ± 0.31	0.07 ± 0.08
Heated sample	0.28 ± 0.36	97.27 ± 0.81	2.59 ± 0.42	0.02 ± 0.03
Eluate A	98.15 ± 0.31	0.17 ± 0.08	1.67 ± 0.21	0.02 ± 0.03
Eluate B	98.13 ± 0.24	0.52 ± 0.51	1.29 ± 0.33	0.05 ± 0.05

Table 3. Distribution of untreated *Chlorella* and treated with heat or eluate from slag referred from earlier reports [7, 9].

It remains unclear why algae in media containing eluate proliferated more than algae in media without eluate (**Figure 5**). In general, the growth and proliferation of photosynthetic organisms, such as land plants and algae, depend strongly on photosynthetic efficiency. Photosynthesis is divisible mainly into two metabolizing systems: light-dependent reactions, which harvest light energy from sunlight and which perform electron transport; and the Calvin cycle, which performs CO_2 fixation to synthesize glucose. This study particularly examined the concentrations of CO_2, which are related to the Calvin cycle, because all experiments in this study were conducted under constant light conditions [11, 13]. This CO_2(aq) can be detected as infrared absorption near 2350 cm^{-1} using FT-IR [11, 13], which is identical to the infrared

absorption attributable to anti-symmetric stretching of CO_2 [39]. To examine the relation between algal proliferation and the concentration of CO_2(aq) under treatment of algae with slag eluate, this study directly evaluated CO_2(aq) in medium containing eluate using FT-IR [11, 13]. The result shows that both eluates had higher infrared absorption identical to CO_2 than that of controls without slag eluate [11, 13]. Next, concentrations of CO_2(aq) under each test condition were quantified from the amounts of total carbonate species using a diaphragm-type electrode to measure CO_2(aq) and from calculation using the Henderson–Hasselbalch equations (**Figure 7**) [11, 13]. The result also showed that concentrations of CO_2(aq) in media containing eluate were higher than those of control samples. Moreover, the concentration in the medium containing eluate B had higher concentrations than that in eluate A. Speculating based on these obtained data, the addition of slag eluate appears to improve aqueous environments for photosynthetic organisms. It might facilitate algal photosynthesis more than CA medium alone. Consequently, increasing concentrations of CO_2(aq) by adding slag eluates induced greater algal proliferation than that in the control sample (**Figure 5**). **Figure 5** shows that this study also stumbled on the fact that the addition of eluate B to the culture medium induced greater proliferation of algae than that of eluate A. Accounting for the different concentrations of CO_2(aq) between eluate A and eluate B, the greater effects of eluate B than those of eluate A on algal proliferation might also be explained.

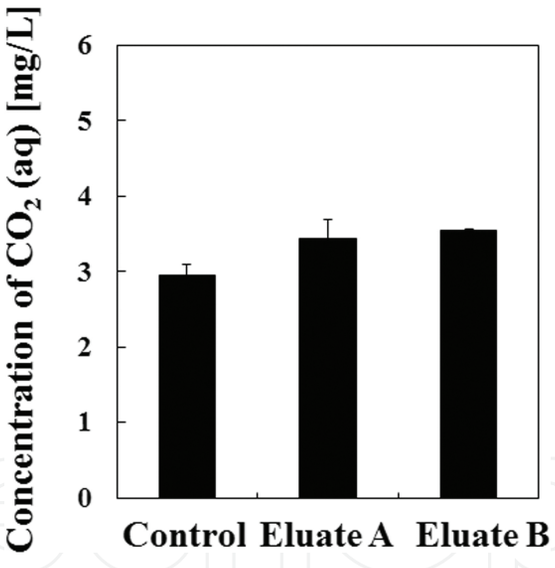

Figure 7. Concentrations of CO_2(aq) in each solution modified from the literature [11, 13]. Eluate A and eluate B, respectively, denote solutions in which concentrations of eluate A and eluate B were 70 vol%.

Formation of the greater concentrations of CO_2(aq) by addition of slag eluate than the control condition has been discussed in the literature [11, 13]. Ca^{2+} and Mg^{2+} are highly reactive substrates with the carbonate species. These elements conveniently existed in higher contents of eluates than other elements (see **Table 2**). As portrayed in **Figure 4**, the fraction of HCO_3^- is the highest content of the carbonate species at pH 7.2, which were experimental conditions used for this study. Therefore, Ca^{2+} in eluate, for instance, might be presumably reacted with HCO_3^- as described in the following Eq. (6).

$$Ca^{2+} + 2HCO_3^- \rightarrow Ca(HCO_3)_2(aq) \quad (6)$$

$Ca(HCO_3)_2$ can interfere in the chemical equilibrium of the carbonate species as HCO_3^- because $Ca(HCO_3)_2$ is ionized completely. Here, the ratios of concentrations of the respective carbonate species must be constant in solution. Consequently, the increase in HCO_3^- concentrations in solution prompts Eq. (2) to proceed leftward, resulting in increasing concentrations of CO_2(aq). Increased CO_2(aq) might be consumed by algae as a raw material of photosynthesis. Assessment of the transitional change of concentrations of Ca^{2+} and Mg^{2+} revealed no significant difference between the concentrations of these elements before and after incubation of algae with eluates, even at 7 days after incubation (**Table 4**). These obtained data support the hypothesis that the addition of slag eluate, particularly Ca^{2+} in eluate, increases the amounts of the total carbonate species and that the increase in the total amounts of the carbonate species by adding slag eluates triggers the potential increase of CO_2(aq), eventually inducing algal proliferation.

	Before incubation	**After incubation**	***p* value (%)**
Concentration of Ca^{2+} (mg/L)			
Eluate A	9.904	8.945 ± 0.917	$p > 0.05$
Eluate B	10.464	9.763 ± 1.056	$p > 0.05$
Concentration of Mg^{2+} (mg/L)			
Eluate A	2.602	3.362 ± 0.381	$p > 0.05$
Eluate B	2.742	2.931 ± 0.075	$p > 0.05$

Table 4. Concentration of alkarin earth elements before and after incubation of *Chlorella* with each eluate, referred from references [7, 9].

The biochemical importance of CO_2(aq) increased by slag eluates was discussed as described in earlier reports [11, 13]. In general, the increase of CO_2(aq) can promote carbon dioxide assimilation by photosynthesis on an algal cellular level. However, the present concentrations of CO_2(gas) in air determine the concentrations of CO_2(aq) that can be dissolved in water. Consequently, the concentrations of CO_2(gas) are regarded as a rate-determining factor of photosynthesis [40]. Its action as the rate-determining factor of CO_2(gas) is common not only to land plants using CO_2(gas) directly but also to aquatic organisms such as phytoplankton using CO_2(aq) dissolved into water. This study indicates that slag components in solution did not cause toxicity to *Chlorella* and that the eluates were able to increase concentrations of CO_2(aq), which functions as the rate-determining factor of photosynthetic organisms in the aqueous environment. This feature of slags in aqueous environment is regarded as beneficial for aqueous photosynthetic organisms including algae. This study was performed using *Chlorella* spp. as the model organism of algae and aqueous photosynthetic organisms. To present the usefulness of slag eluate and their components in algal culture more precisely, additional experiments must be done using photosynthetic organisms other than *Chlorella*.

6. Conclusion

For autotrophic algal biorefineries, biofuel materials, and bioremediation materials, it is important to evaluate features of interesting algae and their qualities in culture. In this study, *Chlorella* was cultured with CA medium containing an eluate from steel slag as an experimental factor and sources of trace metals. Results obtained from this study can be summarized as the following: (1) Slag eluates used for this study met the EQSs for soil pollution, effluent and drinking water, except for Se in eluate A. (2) Analyses of algae treated with the eluate revealed that the eluate from used slag induced neither lethality nor growth inhibition. (3) In addition to cell counting using hemocytometry, bioassay using FCM was able to estimate vigorous and aberrant algal growth simultaneously and graphically. (4) In contrast to comparison of control algae with the heat stress, the distribution of algae treated with the eluate was appropriately similar to that of control, suggesting that the eluate from slags did not give rise to algae stresses. (5) The addition of eluates to the medium increased the concentrations of CO_2(aq). The increased CO_2(aq), which was found to be related to the presence of Ca^{2+} in eluates, improved the rates of photosynthesis and algal proliferation.

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