Evaluation of Metal Toxicity in *Chlorella kessleri* from the Perspective of the Periodic Table

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The toxicities of 33 metals (36 species of metal ions) in *Chlorella kessleri* were investigated and compared to several parameters such as ion radii, stability constants with several ligands, solubility products, and heats of formation (enthalpy). Although a universal parameter that could explain the toxicities of all of the metal ions was not identified, the Irving–Williams series and the HSAB (hard and soft Lewis acidity and basicity) are related to the toxicity of metal ions. With regard to aluminum group elements, the amount of free ion determines the toxicity. Metal absorption was also investigated, including its time dependence (transient absorption). The absorption (adsorption) of anionic species (oxoacid) is lower than that of cationic species which in some cases shows a high collection rate of over 90%. Furthermore, absorptivity varies during the different growth regimes of the cell. Among green alga, *Chlamydomonas reinhardtii* is much more resistant to metal toxicity than *Chlorella kessleri*. Intracellular distribution of zinc was also determined by using a zinc-fluorescent probe under a confocal laser microscope, and the result shows the intracellular distribution of pH could be an important factor for the intracellular distribution of zinc.

Phytoplankton initiates the food-web. It is well known that the formation and dissolution of phytoplankton determine the distribution of metal elements in seawater,[1] i.e., elucidation of the interaction between trace metals and algae may lead to a better understanding of the fate of metal ions in the hydrosphere. Furthermore, phytoplankton has been used for remediation of the aquatic environment. For example, Klimmek et al.[2] evaluated the biosorption of cadmium, lead, nickel, and zinc in 30 strains of algae. Murphy et al.[3] studied copper binding to macroalgae. The importance of bioremediation has been widely recognized; e.g., Radhika et al.[4] investigated *Desulfotomaculum nigrifivans* for use in the elimination of zinc. Surface chemical modification has also been used to increase the biosorption capacity of algae.[5,6] As to the toxicity of metal ions, selected elements have been studied.[7,8] In investigations of phyto-remediation with green algae, comprehensive evaluations of the toxicity and accumulation of metal species against algae are indispensable.

Recently, the biotic ligand model (BLM) has been widely accepted for the assessment of metal toxicity in the aquatic environment, and the effects of metal ions on freshwater organisms can be predicted based on a consideration of the content of dissolved organic carbon, alkalinity, and pH. Di Toro et al.[9] described the fundamental basics of the biotic ligand model, and demonstrated its practical use in the actual aquatic environment.[10] A review up to 2002 has been published by Paquin et al.[11] De Schamphelaere and co-workers applied the biotic ligand model to green algae[12] and green microalgae.[13] This model was developed to explain and predict the acute toxicity of metals toward aquatic organisms.[14–20] The combination of an active metal ion with the biotic ligand site is essential for the generation of metal toxicity. Many studies have focused on copper, where BLM software has been used to predict toxicity.

To determine the nature of the binding site in the BLM, it might be important to investigate the variations in metal toxicity toward algae from the perspective of coordination chemistry. In the present paper, toxicities of 33 metals against *Chlorella kessreli* were examined and compared to inorganic data regarding metal complexation as well as absorption capability to clarify the coordination tendency of biotic ligand in *Chlorella kessreli*. Chlorella has historically been an important target for the examination of metal toxicity. Metal toxicity in *Chlorella kessleri* has been reported previously. Hannan and Patouillet[21] examined the interaction between a specific site on the cell surface and toxicity. Wong and Beaver[22] examined metal toxicity under various conditions including algal competition, species sensitivity, and nutrient levels, and found that Chlorella showed higher tolerance to a mixed-metal solution than to a single metal solution. Kosakowska et al.[23] found that the addition of cysteine was effective at reducing the toxicity of Cu, Cd, and Hg. Wilde et al.[20] showed that both extracellular and intracellular copper were directly related to growth inhibition in Chlorella, whereas zinc toxicity was related to cell-bound zinc.

A review by Worms et al.[24] noted the complexity of metal transport and the availability of trace metals. Exudates for dissolving metal hydroxide precipitates exist in micro-biological systems,[25] and trace metal-bioligand complexes are an example.[26–28] In our previous paper, ammonium complexation at a specific coordination enhanced the uptake of cobalt into bacterial cells.[29] Koukal et al.[25] reported that an algal secretion dissolves metal precipitate in metal transport, which contributes to a reduction in metal toxicity. This means that metals that are present in participates are toxic toward algae. The apparent toxicity (estimation of metal toxicity expressed in terms of the

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amount added) is sometimes used to express the toxicity of metals at an equilibrium between unexpected ligands and metals. Thus, microorganisms should contain non-unique ligands, as pointed out by Worms et al.[24] Notwithstanding the necessity of biological, chemical, and physical processes at the biological interface, the apparent toxicity of metal ions based on amount added is still important for evaluating the effect of metal toxicity in aquatic systems.

Experimental

Test Species. *Chlorella kessleri* C-531, a single-cell fresh water alga, was chosen as the test organism. The test strain was obtained from the IAM Culture Collection at the Institute of Molecular and Cellular Biosciences, the University of Tokyo (Originally the same as 211-11h, Algensammlung der Pflanzenphysiologie, Göttingen). The toxicities of metal ions were compared to those in *Chlamydomonas reinhardtii* CC125 which was also obtained from the Institute of Molecular and Cellular Biosciences.

Cultivation. Chlorella cells were cultivated in Gamborg's B5 medium (commercial solution purchased from Wako Pure Chemical Industries Ltd. diluted 5-fold) to which had been added 5 mM D(+)-glucose (Kanto Chemical Co., Inc.). The medium was diluted 5-fold with 0.02 M HEPES buffer (Wako) and NaOH, 0.005 M ($=$mol L^{-1}) D(+)-glucose was added and the mixture was autoclaved. Metal salts were dissolved in 1% nitric acid at a metal ion concentration of 10 mM, and were used as stock solutions. These stock solutions were diluted with Milli-Q water and were sterilized by filtration with a membrane (cellulose acetate) filter (pore size: 0.20 µm, Iwaki Glass, Japan) before being added to the incubation medium. Incubation was performed at 25 °C under illumination at 3.4 W m^{-2} with shaking at 100 rpm.

Evaluation of Metal Toxicity. Table 1 shows the metal compounds used in this experiment, of which the chloride or nitrate was the major species (Wako). Metal toxicity was evaluated from the growth curve of Chlorella, cells of which were seeded in media containing metal at about 0.5×10^6 cells mL^{-1}, with shaking at 25 °C under illumination at 3.4 W m^{-2}. The growth of cells was monitored by measuring chlorophyll absorption (O.D. 650 nm and O.D. 665 nm) and turbidity (O.D. 730 nm). The pH of the culture after addition of metal solution was adjusted to around 7.0. After 96 h of growth, the O.D. at 730 nm in each vessel was taken, and the 50% inhibitory concentrations (IC_{50}) of the metals tested were evaluated from the inhibition curves. After cells were cultivated for 48 h under the above conditions, the metal ion was added to the culture, and the 50% lethal dose (LD_{50}) for cells in the stationary state was measured after 24 h of incubation, where the decrease in chlorophyll absorption was used as an index of living cells. IC_{50} and LD_{50} values with the standard deviation were calculated based on three independent experiments.

Free metal ion activity was calculated using Visual MINTEQ software (ver. 2.51).[35] In this case, the inhibitory curve against the free metal ion activity was obtained and IC_{50} was obtained. IC_{50} values based on the amount of metal element added were called apparent IC_{50}.

Measurement of Metal Uptake for Cells in the Stationary State. After cells were cultivated for 48 h to reach the stationary state, the metal ion was added and cells were incubated with shaking for 48 h. The cells were collected by centrifugation (3500 rpm, 5 min), and then digested with 20%-H_2O_2 and conc. HNO_3. The digested cell solution was diluted with Milli-Q water and metal uptake was measured with an ICP-AES (SPS 7800, Seiko Instruments Inc., Japan).

Measurement of Metal Uptake in Cells in the Proliferative State. Chlorella cells were seeded at a concentration of 0.5×10^6 cells mL^{-1} into 20 mL of culture medium which contained metal ions, and cultivated for 48 h. The cells were collected and metal content was analyzed by the same method as described for the metal analysis in stationary cells.

Observation of Zinc Distribution in Chlorella Cells. Chlorella cells in the stationary state were exposed to 100 µM zinc for 48 h and were immersed in 10 µM ZnAF-DA (Daiichi Pure Chemicals Co., Ltd., Tokyo) with 100 mM HEPES-NaOH (pH 7.7), and immediately observed by confocal laser scanning microscopy (type TCS SP2, Leica Microsystems, Germany) with excitation at 488 nm emission of an Ar^+ laser. Zinc is the only transition metal that we know of for developing a fluorescent probe (ZnAF-DA: emission $=$ 510 nm), and emission at 680 nm was measured for chlorophyll.

Results and Discussion

Metal Ion Toxicity Expressed as IC_{50}. Figure 1 shows the periodic table with the IC_{50} values of the 33 metals examined. In this figure, IC_{50} values were based on the amount of metal added to the cultivated media. The free ion activity of some elements could not be calculated or was too small (most metal added exists as the hydroxide form). In this case, the IC_{50} is called the apparent IC_{50}. The metal activity (free ion) at the IC_{50} concentration (obtained by the inhibitory curve based on the free ion activity calculated by using Visual MINTEQ software) is given in Table 1 based on the free ion activity model (FIAM) (which is described in Worms et al.[24] or in the references in the manual for Visual MINTEQ[35]). Among the anionic species listed in Table 2, only Cr^{6+} shows activity, while other species exist as poly acids. The most toxic metal is mercury, which has an IC_{50} of 0.36 µM, followed by thallium (3.7 µM). Based on ion activity, Zr^{3+}, Ga^{3+}, In^{3+}, and Sn^{2+} are more toxic than Hg^{2+} and Tl^+. Toxicity as evaluated from the free ion concentration is different than the general conception. In particular, Hg^{2+} and Tl^+ are commonly used as sterilizers or toxins. Zr^{3+}, Ga^{3+}, In^{3+}, and Sn^{2+} exist as hydroxides. Based on a consideration of the mechanism of dissolution described by Koukal et al.[25] or Worms et al.,[24] some portion of hydroxides of these metal ions contributes to the toxicity as evaluated based on the free ion activity. Tables 1 and 2 show the chemical characteristics of cationic metal ions and oxo-acid(anionic) metal ions, respectively. With regard to the characteristics of metal ions that can explain their toxicity toward *Chlorella kessleri*, we considered the ionic radius, stability constants against specific ligands, heat of formation (enthalpy), and solubility product. Table 3 shows the slope of the linear regression between the IC_{50} and various characteristics of the tested metal ions, which is calculated based on the data given in Table 1. The coefficients of dispersion (r^2) from the linear regression line are also given. In metal transport into a cell, the ionic radius (Table 1) is an important factor, i.e., some metal ions that have ionic radii similar to that of a crucial metal ion are taken into the cell via the same transport systems. However, in the present experiment, a very low correlation was found between IC_{50} and the metal ion radii based on both activities and the apparent amount. In detail, the slope changes from negative to positive and dispersion (r^2) becomes about 40

Table 1. Chemical Constants for the Metal Ions Examined (Cations)[a),b)]

					Stability constant								
		IC_{50}[c)]	Activity	Ion radius[d)]	Bipyridyl	*o*-phen	NH_3	H_2S	$H_2C_2O_4$	CH_3COOH	Oxide: ΔH	Sulfide: ΔH	log K_{sp} (Sulfide)
Sc^{3+}-6	$ScCl_3$	80.1	0.00234	0.75					6.86		−1908.82		
Y^{3+}-6	YCl_3	240	3.46	0.9					5.46	1.59	−455.38		
Ti^{3+}	$TiCl_4$	193	0	0.67							−394.6		
Zr^{4+}	$ZrCl_4$	1550	<4.59E−19	0.72							−1100.56	−566.1	
V^{3+}	VCl_3	146	1.19E−05	0.64							−1218.8		
Nb^{+}	$NbCl_5$	183		0.72							−1899.5		
Cr^{3+}	$Cr(NO_3)_3 \cdot 9H_2O$	85.3	0.00077	0.62						4.64	−1139.7		
Mn^{2+}	$MnCl_2 \cdot 4H_2O$	183	98.3	0.83	2.62	4	1	−10.5	3.95	1.4	−385.2	−214.2	−9.6
Fe^{2+}	$FeCl_2 \cdot 4H_2O$	183	95.7	0.61	4.65	5.85	2.81	−18.1	3.05	1.4	−272	−100	−17.2
Ru^{3+}	$RuCl_3$	18.4		0.68							−72.9	−47	
Co^{2+}	$CoCl_2 \cdot 6H_2O$	118	56	0.65	5.81	7.08	2.1	−21.3	3.25	1.46	−237.9	−82.8	−20.5
Rh^{3+}	$RhCl_3$	8.6		0.67							−343		
Pd^{2+}	$PdCl_2$	13.3	7.76E−11	0.86			9.6				−85.4	−75	
Ni^{2+}	$Ni(NO_3)_2 \cdot 6H_2O$	70.5	31.4	0.69	7.06	8.6		−24.9	5.16	1.43	−385.2	−214.2	−18.5
Pt^{4+}	$PtCl_4$	9.7		0.63								−108.8	
Cu^{2+}	$CuSO_4 \cdot 5H_2O$	25.8	2.08	0.73	6.33	7.4	4.24	−48.5	6.23		−157.3	−53.1	−35.2
Zn^{2+}	$ZnSO_4 \cdot 7H_2O$	101	58.2	0.74	5.13	6.73	2.28	−24.7	4.87	1.57	−348.28	−193.21	−21.6
Cd^{2+}	$CdCl_2 \cdot 2.5H_2O$	32.1	6.89	0.95	4.18		2.72	−27	3.89	1.93	−258.4	−161.9	−26.1
Hg^{2+}	$HgCl_2$	0.36	1.06E−10	1.02	9.64		8.8	−53.3	9.66	5.55	−90.8	−58.2	−53.4
Al^{3+}	$Al(NO_3)_3 \cdot 9H_2O$	435	3.00E−03	0.54					6.1	1.51	−1675.7	−724	−6.7
Ga^{3+}	$GaCl_3 \cdot 3H_2O$	27.5	6.10E−11	0.62	4.52	5.58			6.45		−1089.1		
In^{3+}	$In(NO_3)_3 \cdot 3H_2O$	155	3.20E−07	0.8	4.75	5.7		−69.4	5.3	3.5	−925.8	−427	
Tl^{+}	$TlNO_3$	3.7	2.89E+00	1.5			−0.9	−21.2		−0.11	−178.7	−97.1	−20.3
La^{3+}	$La(NO_3)_3 \cdot 6H_2O$	313	5.88E+01	1.03					4.3	1.59	−1793.7	−1209	−12.7
Eu^{3+}	$Eu(NO_3)_3 \cdot 6H_2O$	287	53.5	0.95					5.04	1.9	−1651.4		
Ge^{4+}	$GeCl_4$	860		0.53							−580	−121.5	
Sn^{2+}	$SnCl_2$	717	8.87E−05	1.18						3.3	−280.71	−100	−25
Sn^{4+}	$SnCl_4 \cdot 5H_2O$	911	9.30E−19	0.69							−577.63		
Pb^{2+}	$Pb(CH_3COO)_2 \cdot H_2O$	359	146	1.19	2.9	4.65		−27.5		2.68	−219	−100.4	−27.1

a) Ionic radii are given for a coordination number of 6 for the transition and aluminum group metal ions. S.C.: stability constant ($M^{n+} + L = M^{n+}L$), *o*-phen: 1,10-phenanthroline, Enthalpy: enthalpy (heat) of formation for MxSy or MxOy. K_{sp}: solubility product, ΔH: enthalpy (kJ mol^{-1}). b) The second column shows the compounds used as the metal ion source in the present study. The data except for the stability constants were taken from Refs. 30–34. c) IC_{50} is indicated as µM. d) Ion radius is indicated as 0.1 nm.

Periodic Table of the Elements

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	H																	He
2	Li	Be											B	C	N	O	F	Ne
3	Na	Mg											Al 435(III)	Si	P	S	Cl	Ar
4	K	Ca	*Sc 80.1(III)	*Ti 193(IV)	*V 146(III) 146(V)	*Cr 85.3(III) 23.0(VI)	*Mn 183(II)	*Fe 183(II)	*Co 118(II)	*Ni 70.5(II)	*Cu 25.8(II)	Zn 101(II)	Ga 27.5(III)	Ge 859(IV)	As 868(III) 2.84x10^{3}(V)	Se	Br	Kr
5	Rb	Sr	*Y 240(III)	*Zr 1.55x10^{3}(IV)	*Nb 183(V)	*Mo 12.7x10^{3}(VI)	*Tc	*Ru 18.4(II)	*Rh 8.6(II)	*Pd 13.3(II)	*Ag	Cd 32.1(II)	In 155(III)	Sn 717(II) 912(IV)	Sb 8.17x10^{3}(V)	Te	I	Xe
6	Cs	Ba	Lanthanoids	*Hf	*Ta	*W 8.3x10^{3}(VI)	*Re	*Os	*Ir	*Pt 9.7(II)	*Au	Hg 0.36(II)	Tl 3.7(I)	Pb 359(II)	Bi	Po	At	Rn
7	Fr	Ra	Actinoids															

Lanthanoids	*La 313(III)	*Ce	*Pr	*Nd	*Pm	*Sm	*Eu 287(III)	*Gd	*Tb	*Dy	*Ho	*Er	*Tm	*Yb	*Lu
Actinoids	*Ac	*Th	*Pa	*U	*Np	*Pu	*Am	*Cm	*Bk	*Cf	*Es	*Fm	*Md	*No	*Lr

* Transition Element

Figure 1.

times greater (Table 3) when the free metal activity is taken from the apparent amount. Regarding the ionic radius, it has been pointed out[36] that the positive metal ions in lower positions in the same group in the periodic table show stronger toxicity. Thus, larger (and heavier) metal ions in the same vertical group in the periodic table have greater toxicity, and this trend was not seen in the apparent base correlation (slight negative slope and low dispersion coefficient). However, in the aluminum group, the apparent IC_{50} for Ga^{3+} is 16 and 6 times lower than those of Al^{3+} and In^{3+}, respectively. This irregularity is resolved when taking IC_{50} based on the free ion activity. Strong toxicity (in apparent IC_{50}) of Tl^{+} should be due to the large free ion amount among the aluminum group. The single charge of thallium prevents the formation of hydroxide compared to other aluminum group metal ions which possess a triple charge. Aluminum group ions are typical examples of FIAM. The LC_{50}/IC_{50} ratio in free ion base simply decreased from aluminum to thallium, showing the appearance of the heavier metal ion toxicity for the growth stage in Chlorella.

Table 2. Chemical Species and Their Redox Potentials for Metal Ions (Anions)[a)]

		IC_{50}[b)]	Redox potential/V
As^{3+}	$NaAsO_2$	868	−0.68
As^{5+}	$Na_2HAsO_4 \cdot 7H_2O$	2836	−0.67
V^{5+}	$NaVO_3$	146	0.542
Cr^{6+}	$Na_2Cr_2O_7 \cdot 2H_2O$	23	1.36
Mo^{6+}	$Na_2MoO_4 \cdot 2H_2O$	12700	−0.913
W^{6+}	$Na_2WO_4 \cdot 2H_2O$	8300	−1.074
Sb^{5+}	$K[Sb(OH)_6)]$	8170	−0.6389

a) The second column shows the compounds used as the metal ion source in the present study. The value in IC_{50} is based on the apparent value. The data were taken from *Lange's Hand Book of Chemistry*, **2005**. b) IC_{50} is indicated as μM.

With regard to the apparent IC_{50}, a good relationship was found between the metal toxicity and the stability constant for ligands in which the coordination atom is nitrogen, such as 1,10-phenanthroline and bipyridine, where the slope (negative) and regression coefficient are both high. On the other hand, if we consider the free metal ion activity, improved regression coefficients with negative slopes were obtained in the correlation graphs of the stability constant for H_2S (S^{2-}), heat of formation (enthalpy) and solubility product for sulfide. It is clear that binding to sulfur is closely related to the appearance of free metal ion toxicity. Typical regression lines are shown in Figure 2. From the perspective of BLM, the toxic site in Chlorella shows sulfur coordination nature. The affinity factors to sulfur, solubility product and heat of formation were also taken. In these factors, the toxicities based on free ion activity also showed negative slope in the correlation graphs. Furthermore, sulfide ion is an important factor in the environmental water in the BLM theory. The internal sulfur in the cell might also play a role in the metal ion toxicity. In most textbooks, soft metals exhibit high toxicity, and sulfur is a representative soft ligand. Mercury and thallium show high toxicity, and are also representative soft metals. Therefore, metal affinity to sulfur also is important in the toxicity. Recently, Miao and Wang[37] pointed out that the synthesis of intracellular cysteine and some thiols does not alleviate copper toxicity. Thus, direct interaction between sulfur (a representative soft coordinating element) in the intracellular thiols might provide contribution in the expression in the toxicity of metal ions. We have found that lanthanide ions (here we examined La^{3+} and Eu^{3+}) promote the aggregation (and precipitation) of Chlorella cells before fatal damage. This phenomenon is due to their high valences and rather large ionic radii.

Table 3. Slope and Dispersion of the Regression of IC_{50} and Chemical Factors

	Ion radius	S.C.[a] H_2S	S.C.[a] $H_2C_2O_4$	S.C.[a] Acetic acid	S.C.[a] *o*-phen	S.C.[a] bipyridine	S.C.[a] NH_3	Enthalpy Sulfide	Enthalpy Oxide	K_{sp}[b] Sulfide
Based on the apparent value										
Inclination	−0.00004	+0.035	−0.0038	+0.0002	−0.0087	−0.0124	−0.0201	−0.250	−0.296	+0.0135
Dispersion (r^2)	0.0043	0.0435	0.0815	0.0007	0.391	0.427	0.188	0.11	0.029	0.115
Based on the activity										
Inclination	0.0107	−1.1948	0.0253	0.052	−0.0398	−0.0193	−0.0144	−4.9137	8.5314	−0.3559
Dispersion (r^2)	0.1697	0.1813	0.0131	0.0146	0.0362	0.0037	0.0004	0.0133	0.0146	0.0382

a) S.C.: stability constants ($\log K$). b) K_{sp}: solubility product.

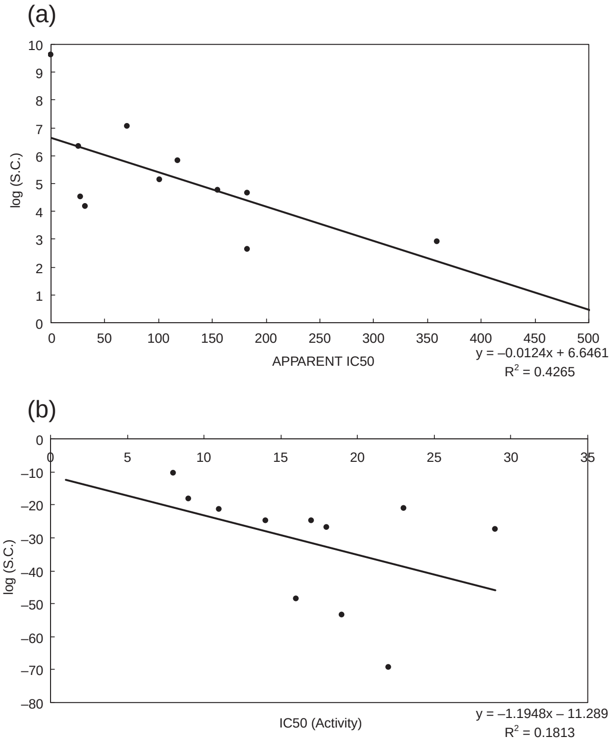

Figure 2. Examples of the best regression line. (a) Correlation between apparent IC_{50} and log(S.C.) for bipyridine. (b) Correlation between IC_{50} based on activity and log(S.C.) for H_2S. S.C. = stability constant.

The Irving–Williams series, which is a sequence of the general stability of high-spin octahedral complexes of M^{2+} ions in the first transition metals, is consistent with the apparent and activity based IC_{50} values; Mn $<$ Fe $<$ Co $<$ Ni $<$ Cu $>$ Zn. We can conclude the general trend in metal toxicity follows the complexation due to the Irving–Williams series.

Table 2 also shows the toxicities (IC_{50}) of oxoacid metal ions. For most species it is impossible to calculate activity. However, these anionic metal complexes show weak toxicity (on the basis of their apparent IC_{50} value) compared to the cationic metal complexes, except for chromate (Cr^{6+}) and vanadate (V^{5+}). These ions possess high redox potential (Table 2). The strong oxidizing power of these oxoacids is expressed as high toxicity. Corradi et al.[38] pointed out that the sensitivity of algae to Cr^{6+}, is related to the reduction of Cr^{6+} to Cr^{3+} due to exudates of algae. In addition to the extracellular reduction of Cr^{6+},[39] the transport of Cr^{6+} into the cell, reduction to Cr^{3+}, and reaction with intracellular material has been known for several decades (for example Martell[40] and Shen and Wang[41]). Metals that show a negative charge are scarcely taken into cells. The surface of *Chlorella kessleri* is known to have a negative charge (for example, Svetlicic and Hozic[42]). Electrostatic repulsion reduces the intake of these ions, resulting in their low toxicity, except Cr^{6+} and V^{5+}. Indeed, molybdate and tungstate are almost nonpoisonous.

Comparison of IC_{50} and LC_{50}. Tables 4 and 5 show the LC_{50}/IC_{50} ratios for cations and anions, respectively. All of the metal species show an LC_{50}/IC_{50} ratio of more than 1, which suggests that grown cells are more resistant to metal toxicity than those that are still growing. However, except for some species (Cu^{2+}, Ga^{3+}, In^{3+}, Tl^{+}, VO_3^{-}, and $Cr_2O_7^{2-}$), most metals show a ratio of less than 10 (around 5). In the cell cycle of *Chlorella kessleri*, a mature cell (stationary phase) is about 5 times more resistant than a nascent cell (proliferative phase) based on the apparent values. This difference may be due to the effect of metal in two different processes, i.e., maintenance of chloroplasts (phytotoxicity: in this study, LC_{50} was evaluated based on chlorophyll absorption) and cell duplication. Interestingly, chromate is not very poisonous to cells in the stationary phase and shows a high LC_{50} value. Including molybdate and tungstate (which are less-poisonous metal species), the difference between LD_{50} and IC_{50} for negatively charged metal species is between 4 and 20. These results are similar to those for positively charged metal ions. On the basis of activity, the LC_{50}/IC_{50} ratio was not different from those based on the apparent LC_{50} and IC_{50} values except for Sc^{3+}, Ti^{4+}, V^{3+}, and Tl^{+}. Most of these exceptional species provide higher values based on free ion activity than based on apparent amount. It is reasonable that the activity decreases with an increase in the metal concentration, especially for species of higher valence.

Absorption of Metal Ions in Chlorella Cells. Figure 3 shows the proportion of metal ions (cation) absorbed into cells in the stationary phase. Although there is a difference in the concentration range of metal ions, Chlorella cells show high absorptivity of close to 100% for aluminum group metals.

Table 4. LC_{50} and LC_{50}/C_{50} Ratio for Cationic Ions

	LC_{50}a)	Activity	LC_{50}/IC_{50} (Apparent value)	LC_{50}/IC_{50} (Activity)
Sc^{3+}	420	0.0272	5.24	11.6
Y^{3+}	—b)		—b)	
Ti^{3+}	591	—c)	3.06	>10000
Zr^{4+}	—b)		—b)	
V^{3+}	572	—d)	3.92	>10000
Nb^{5+}	—b)		—b)	
Cr^{3+}	780	0.00887	9.14	11.52
Mn^{2+}	745	409	4.07	4.15
Fe^{2+}	847	447	4.63	4.67
Ru^{3+}	93		5.05	
Co^{2+}	723	408	6.13	7.29
Rh^{3+}	12.5		1.45	
Pd^{2+}	39	2.36E−9	2.93	43.3
Ni^{2+}	458	408	6.5	13.0
Pt^{4+}	54.5		5.62	
Cu^{2+}	481	103	18.6	49.5
Zn^{2+}	529	286	5.24	4.91
Cd^{2+}	1200	625	37.4	90.7
Hg^{2+}	—b)		—b)	
Al^{3+}	780	0.005	1.79	1.66
Ga^{3+}	5200	1.78E−8	189	2818
In^{3+}	6900	1.69E−5	44.5	52.8
Tl^{+}	160	141	43.2	48.7

a) LC_{50} is indicated as µM. b) LC_{50} was not determined. c) Ti = $Ti(OH)_4$—100%. d) V = $V(OH)_3$—100%.

Table 5. LC_{50} and LC_{50}/IC_{50} Ratio for Anionic Ions

	LC_{50}a)	LC_{50}/IC_{50}	Activity	LC_{50}/IC_{50} (Activity)
As^{3+}	—b)	—b)		
As^{5+}	—b)	—b)		
V^{5+}	580	3.97		
Cr^{6+}	526	22.9	251	22.6
Mo^{6+}	80×10^3	6.3		
W^{6+}	80×10^3	9.64		
Sb^{5+}	—b)	—b)		

a) LC_{50} is indicated as µM. b) LC_{50} was not determined.

Among the first-transition metals, less than 10% of early metals, such as Cr^{3+}, Mn^{2+}, Fe^{2+}, and Co^{2+}, are absorbed in the concentration range examined. On the other hand, more than 20% of the late metals, such as Ni^{2+}, Cu^{2+}, and Zn^{2+}, are absorbed. The late metal ions have slightly larger radii than the early metal ions. Thus, absorption among the first-transition metals might be limited by the ionic radius. The metal concentration (abscissa) shows the apparent value (amount added to the medium), which means that the metal hydroxide (found in the aluminum group) was adsorbed more compared to the free ion. It is clear that metal adsorptivity is not related to metal toxicity among positive ions. For example, Al^{3+} is more adsorbed (less toxic) than the first transition metal ions are (more toxic). De Schamphelaere et al.[12] descibed the relationship between toxicity and metal ions taken into the cell. The participation of a transport system that transports into the cell is an essential factor in toxicity, as pointed out by Worms et al.[24] Furthermore, Koukal et al.[25] reported the dissolution of hydroxide aggregate by exudates from algae and the uptake of hydroxide with participation of chelaters including siderophore. In the present study, we did not distinguish between metal inside the cell and metal adsorbed to the peripheral cell wall. However, based on the time-dependent adsorption (absorption) of aluminum group metal or chromium (see Figure 5), an appreciable amount of these metals is transported inside the cell.

In the case of negatively charged ions (oxoacid form) shown in Figure 4, absorption is very low and it is possible that this low interaction between Chlorella cells and metals is responsible for their low toxicity, except for chromate. This situation can be explained according to the electric repulsion between the oxo-formed metal ion and the cell surface (possessing minus charge[43,44]). Among metals found in the sea, Mo and W are almost constant (or show very low variation) in various areas, and these metals are so-called preservative elements.[1] It is well known that phytoplankton play an important role in the distribution of trace metals in the sea. Little interaction between Chlorella and molybdate or tungstate was observed in the present study. Due to the high toxicity of Cr^{6+}, the nature (including acclimation) of its bio-sorption has been studied.[45,46] With regard to the bio-sorption of Cr^{6+}, complexation with an amino group at the cell wall has been pointed out in the case of the fungi.[6]

There are many studies that point out the plural existence of metal ion transporters. For example, divalent metal transporters were examined for Mn^{2+}, Fe^{2+}, Cu^{2+}, and Cd^{2+}.[47] Also, arsenite uptake into the cell is via the transporter of phosphate.[48] Alvarez et al. discussed the Cr^{4+} transporter for Pseudomonas.[49] Some of the metal transporters are also related to the growing condition of algae. Therefore, it should be considered that the intracellular concentration of each metal ion is dependant on the activity of the respective metal transporters. Further study is required to clarify the determining factors affecting intracellular metal concentration.

Absorption of Metal Ions in Different Growth Regimes. Table 6 shows the adsorption (absorption) of metal ions during cell growth in *Chlorella kessleri*, i.e., metal adsorption is compared in the proliferative and stationary phases. Although there are some differences in the concentrations of metal ions, positively charged metal ions show 3- to 7-fold greater adsorption (absorption) in proliferative cells than in stationary cells. The fact that there is greater uptake of metal ions during the growth (proliferative) phase may be related to the finding that IC_{50} is less than LC_{50}. Metal ions in oxoacid form (negatively charged) are clearly absorbed more in the stationary phase than in the proliferative phase. This might be related to the charge on the cell surface in the proliferative (growth) and stationary phases.

Time-Dependence of Metal Ion Absorption. Figures 5–7 show the time-dependence of metal ion absorption in Chlorella cells in the stationary phase for Cr^{3+}, Zn^{2+}, Cd^{2+}, Al^{3+}, Ga^{3+}, In^{3+}, Tl^{+}, CrO_4^{2-}, MoO_4^{2-}, and WO_4^{2-}. Among these species, Cr^{3+}, Al^{3+}, Ga^{3+}, MoO_4^{2-}, and WO_4^{2-} were quickly absorbed (adsorbed) to the cell, reaching 90% of their maximum absorptions (adsorptions) within 30 min. In the case of Zn^{2+} at

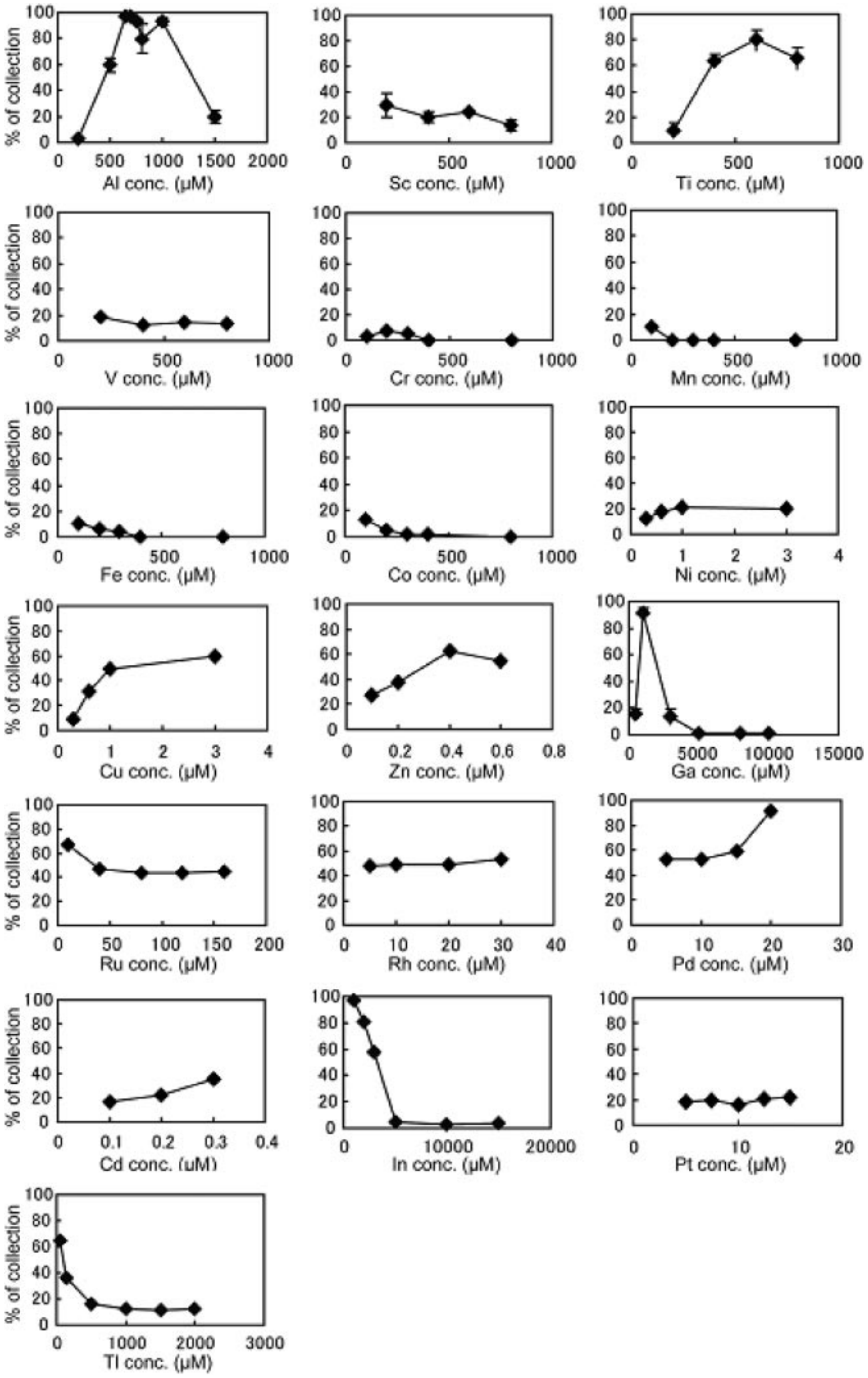

Figure 3. Collection efficiencies (proportion of absorption) of metal ions added to culture (cations).

pH 5.8, absorption increased almost linearly with time up to 3 h and then gradually decreased after 9 h. Özer et al.[50] described two processes in zinc absorption in green algae (Steps 1 and 2). Step 1 is fast and is caused by physicochemical interactions, including adsorption to polysaccharides and glycoproteins located in the cell wall and ion exchange on the cell surface. On the other hand, Step 2 is slow and zinc is taken into the cell via a metal transport system. The difference in Zn^{2+} absorption at pH 5.8 and 7.8 shown in Figure 4 suggests that initial absorption (linear increase in uptake with time) occurred within 1 h at pH 5.8, but persisted for 3 h at pH 7.8. In this paper, this initial increase in metal absorption is also categorized as Step 1, and the subsequent slow increase is considered Step 2. A decrease in pH reflects an increase in competition in the replacement of H^+ and zinc ions in the cell wall, resulting in the prolongation of Step 1 at lower pH. Step 2 was observed under both pH conditions. This process has a hard nature in the HSAB theory. The hardness of a metal ion as a Lewis acid can explain the fact that Cd^{2+} gives a slower appearance of the Step 1 than Zn^{2+}, where Cd^{2+} is a softer acid than Zn^{2+}. Regarding the time-dependent uptake of zinc in *Chlorella kessleri*, Hassler et al.[51] described the importance

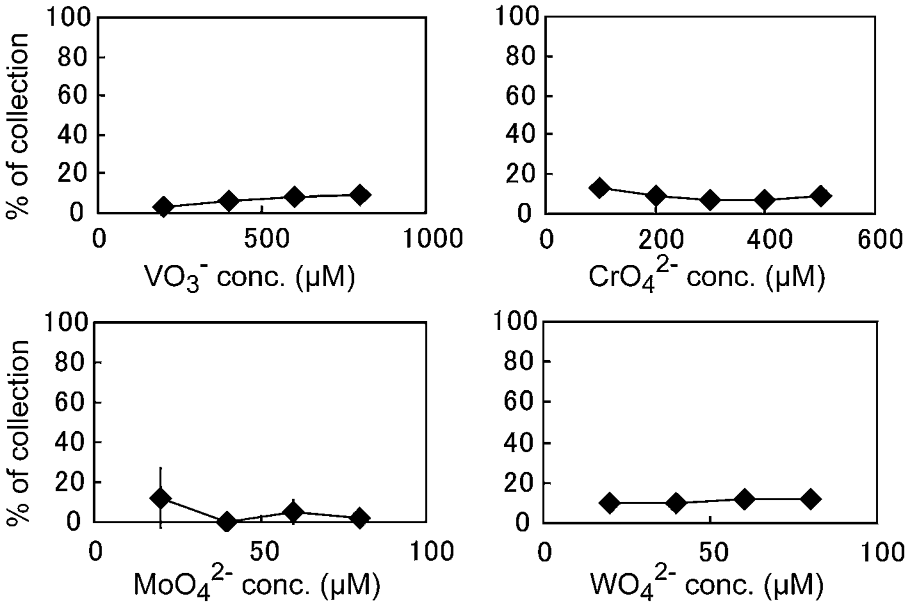

Figure 4. Collection efficiencies (proportion of absorption) of metal ions added to culture (anions).

Table 6. Adsorption (Absorption) Efficiency of Metal Ions to Chlorella Cells when Added to the Culture

Proliferative phase		Retrieval/%	Stationary phase		Retrieval/%
Cr^{3+}	25 μM	31.0 ± 1.2	Cr^{3+}	100 μM	3.13 ± 2.4
	50 μM	38.3 ± 1.9		200 μM	6.92 ± 1.1
	75 μM	35.6 ± 1.8		300 μM	5.12 ± 0.1
				400 μM	0.31 ± 0.3
				800 μM	0.01 ± 0.00
Mn^{2+}	50 μM	8.1 ± 0.7	Mn^{2+}	100 μM	9.89 ± 5.4
	100 μM	7.9 ± 1.2		200 μM	0.39 ± 4.0
	150 μM	1.8 ± 0.1		300 μM	0.30 ± 0.2
	200 μM	1.8 ± 0.0		400 μM	0.00 ± 0.4
				800 μM	0.03 ± 0.1
Fe^{2+}	50 μM	28.7 ± 2.7	Fe^{2+}	100 μM	10.50 ± 6.7
	100 μM	25.6 ± 1.8		200 μM	6.07 ± 4.0
	150 μM	2.3 ± 0.1		300 μM	4.62 ± 0.3
	200 μM	3.3 ± 0.0		400 μM	0.00 ± 0.4
				800 μM	0.03 ± 0.1
Co^{2+}	50 μM	2.3 ± 0.6	Co^{2+}	100 μM	12.8 ± 2.9
	100 μM	2.1 ± 0.3		200 μM	5.10 ± 1.4
	150 μM	1.8 ± 0.3		300 μM	2.42 ± 0.7
	200 μM	1.8 ± 0.2		400 μM	1.81 ± 0.5
				800 μM	0.44 ± 0.3
Cr^{6+}	50 μM	2.0 ± 1.0		50 μM	16.0 ± 2.0
Mo^{6+}	1000 μM	0.19 ± 0.05		1000 μM	13.0 ± 5.0
W^{6+}	1000 μM	0.016 ± 0.006		1000 μM	9.0 ± 1.0

of homeostasis; the system maintained a constant zinc content in the cell interior. An example is shown in Figure 8. Under light (photosynthetic condition), intracellular distribution of zinc is the same as that of chlorophyll. The pH of the chloroplast increases and reaches close to 9.0. This means zinc taken into the cell interior was concentrated in the chloroplast as in the form of hydroxide. The uptake of metal ions into the cell interior is related to the physiological condition of the cell, as reflected in the uneven distribution of metal within the cell, as can be seen with zinc. On the supposition that the metal ion toxicity presents at the binding site or location of metal in the cell, intracellular localization of the metal distribution must be important. Unfortunately, the available fluorescence proof is, at present, limited to zinc among the transition metals. When further developments provide proof of the localization of other metal ions, the mechanisms of metal ion toxicity will become increasingly clear on the bases of intracellular metal distribution in the biotic ligand model. In the aluminum group,

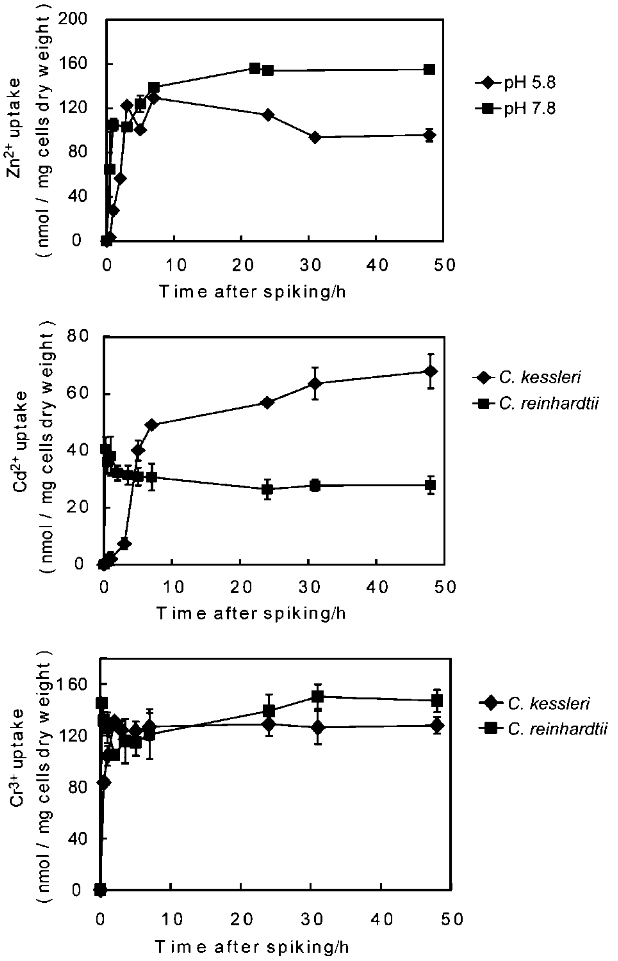

Figure 5. Transient absorption of metal ions to cells in the stationary phase. Spiked concentration: $Zn^{2+} = 15\,\mu M$, $Cd^{2+} = 8.9\,\mu M$, $Cr^{3+} = 19\,\mu M$. Upper figure: Transient absorption of Zn^{2+} for different pH (*Chlorella kessleri*). Middle figure: Transient absorption of Cd^{2+} for different algae (*Chlorella kessleri* and *Chlamydomonas reinhardtii*). Lower figure: Transient absorption of Cr^{3+} for different algae (*Chlorella kessleri* and *Chlamydomonas reinhardtii*).

Al^{3+} and Ga^{3+} showed maximum absorption within 15 min. In the case of In^{3+}, the amount of metal absorbed in the fast (initial) process, which occurs within 15 min, was about 30% of the maximum at 1 h, and the content of thallium then gradually decreased for up to 48 h. There may be some mechanism for the excretion of thallium retained inside the cell.

The high toxicity of thallium ion toward Chlorella may affect the transport of metal ion into the cell. Knauer et al.[52] described two steps in the absorption of Cu^{2+} in green algae, i.e., rapid adsorption to the cell wall was followed by transport inside the cell via the membrane metal transporter. In a preliminary examination for Cr^{3+}, a time-dependent (transient) difference was noted in the absorption of chromium to the cell surface and intracellular sites based on a method proposed by Knauer et al.[52] and Meylan et al.[16] The amount of intracellular chromium gradually increased after a rapid initial increase after the cultivation medium was spiked with chromium.

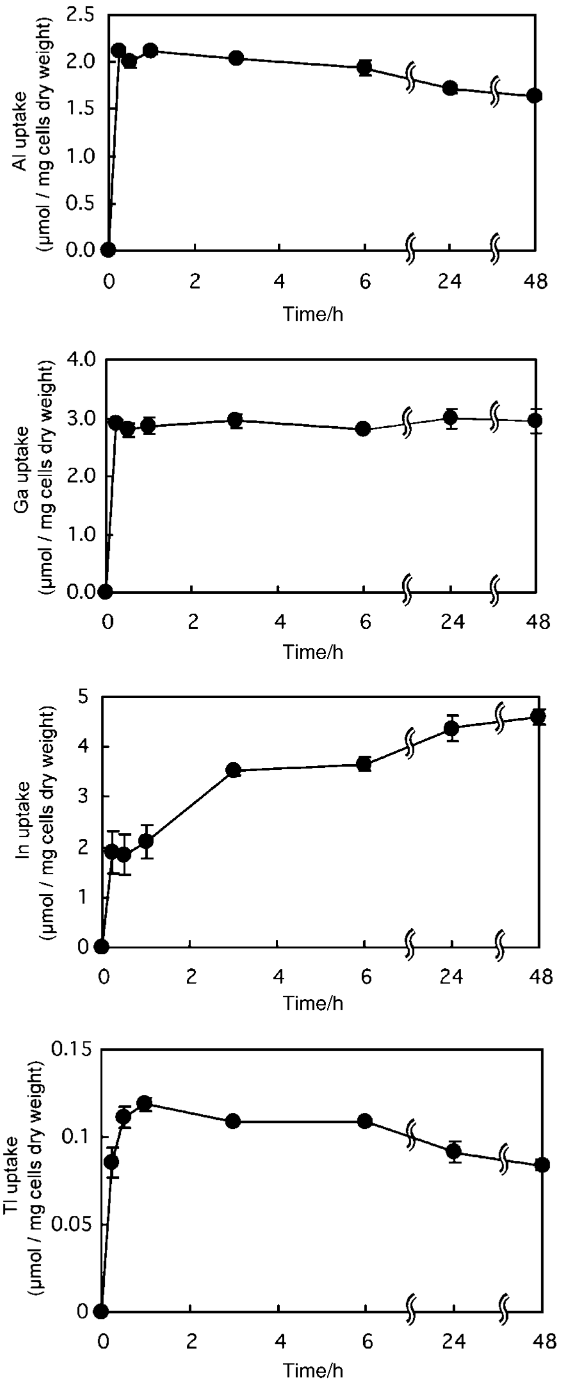

Figure 6. Transient absorption of metal ions to the cell at the stationary phase. Spiked concentration: $Al^{3+} = 0.7\,\mu M$, $Ga^{3+} = 1.0\,\mu M$, $In^{3+} = 2.0\,\mu M$, $Tl^{+} = 0.05\,\mu M$.

The absorption of anionic species (oxoacid of metal) does not provide a Step 1 process as shown in Figure 6. In the case of Cr^{6+}, absorption is low and slow compared to other anionic species: 50% of maximum absorption was achieved within 1 h, and absorption then proceeded gradually over 48 h to maximum (defined as 100%) absorption.

Comparison to Other Green Algae. We compared *Chlamydomonas reinhardtii* CC 125 to *Chlorella kessleri* with respect to the absorption of metal ions. *Chlamydomonas reinhardtii* is also a single-cell freshwater green alga with two flagella. It is similar in size to *Chlorella kessleri*. Table 7 com-

Table 7. Comparison of IC_{50} Values (μM) for *Chlorella kessleri* and *Chlamydomonas reinhardtii*

	Cr^{3+}	Co^{2+}	Ni^{2+}	Cu^{2+}	Cd^{2+}
Based on the apparent value					
Chlorella kessleri	85 ± 9	118 ± 9	71 ± 0.7	26 ± 4	32 ± 3
Chlamydomonas reinhardtii	347 ± 12	170 ± 16	97 ± 3	140 ± 4	150 ± 9
Based on the free ion activity					
Chlorella kessleri	0.0011	56.0 ± 4.0	31.4 ± 0.3	2.1 ± 0.1	6.9 ± 0.6
Chlamydomonas reinhardtii	0.0024	21.3 ± 2.0	1.28 ± 0.1	2.8 ± 0.1	21.3 ± 1.2

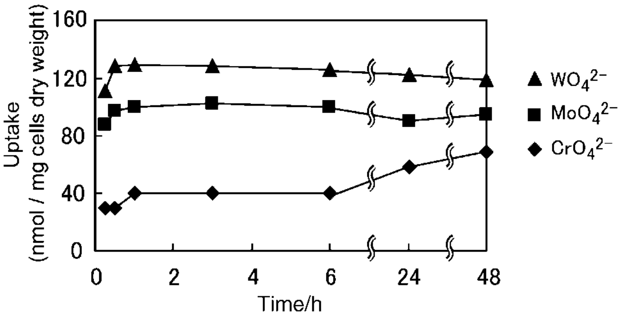

Figure 7. Transient absorption of metal ions to cells in the stationary phase. Spiked concentration is 400 μM for all of the metal species shown.

pares apparent IC_{50} for *Chlamydomonas reinhardtii* and *Chlorella kessleri*. As shown, *Chlamydomonas reinhardtii* is much more resistant to the metal ions examined than *Chlorella kessleri*. As can be seen in Figure 5, the time-dependent uptake of Cd is greater in *Chlorella kessleri* than *Chlamydomonas reinhardtii*. Hiriant-Baer et al.[18] compared *Chlamydomonas reinhardtii* with *Pseudokirchneriella subcapitata*. They examined the effect of the coexistence of thiosulfate, and anionic thiosulfate–silver complex was transported into both algal cells via a membrane-bound sulphate transporter. Despite the higher rate of uptake in *Pseudokirchneriella subcapitata*, *Pseudokirchneriella subcapitata* is more tolerant than *Chlamydomonas reinhardtii*. De Schamphelaere and Janssen (2006) examined the EC_{50} for *Pseudokirchneriella subcapitata* and *Chlorella vulgus* under different pH conditions, and found that the pH-dependencies of the EC_{50} of both algae show a similar slope. Thus, the present results suggested that *Chlorella kessleri* might have more active sites than *Chlamydomonas reinhardtii*.

Conclusion

In the present study, we examined toxicities of more than 30 metals. In some cases, toxicity can be explained by a single chemical factor. For example, the order of toxicity among divalent first-transition metal ions coincides with the Irving–Williams series. Generally, heavier metal ions with large ionic radii are more toxic than lighter ions with smaller ionic radii in the same group of the periodic table. Also, metals in oxoacid form show only weak toxicity that is related to the uptake amount. Regarding the active site in Chlorella (M-Biotic Ligand in Figure 1 of Ref. 8, sulfur coordination is related to the expression of toxicity. In the case of the aluminum group (Al, Ga, In, and Tl), thallium has a single charge and shows high free ion activity, resulting in strong toxicity. It is clear that the activity of metal ion is directly related to binding of the biotic ligand (or presentation of toxicity). For the purpose of understanding metal ion toxicity to algae on the basis of the periodic table, the characteristic of biotic ligand in algae should be comprehensively evaluated on the basis of inorganic or complex chemistry.

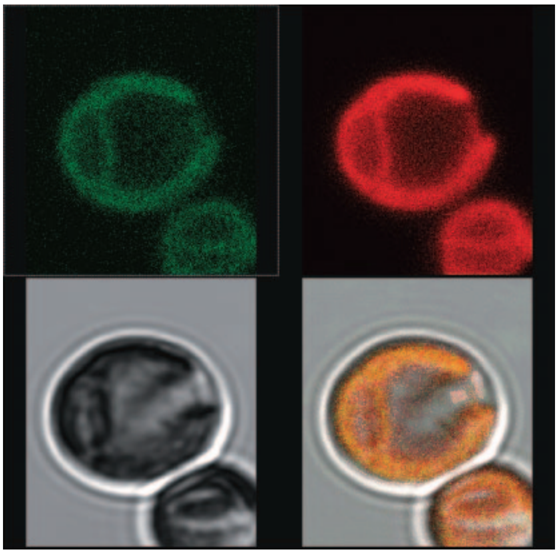

Figure 8. Photos of *Chlorella kessleri* taken with a confocal laser microscope. Upper left: zinc distribution (emission = 510 nm). Upper right: chlorophyll distribution (emission = 680 nm). Lower left: *Chlorella kessleri* (ordinary microscope conditions). Lower right: composite figure of emerging the upper two photos.

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