Effect of nitrate on the determination of iron concentration in phytoplankton culture medium by liquid scintillation counting (LSC) method using 55Fe as radioisotope tracer

doi: 10.1007/s10967-012-2327-4

Effect of Nitrate on the Determination of Iron Concentration in Phytoplankton Culture Medium by Liquid Scintillation Counting (LSC) Method Using ^{55}Fe as Radioisotope Tracer

Chikako Okumura[1], M. Azizur Rahman[1, 2], Akira Takimoto[4], Hiroshi Hasegawa[3,*]

[1]Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa, 920-1192, Japan

[2]Centre for Environmental Sustainability, School of the Environment, University of Technology Sydney, PO Box 123, Broadway, NSW 2007, Australia

[3]Institute of Science and Engineering, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan

[4]Institute of Science and Engineering, Faculty of Mechanical Engineering, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan

[*]**Corresponding author**

E-mail: hhiroshi@t.kanazawa-u.ac.jp (H. Hasegawa)

Tel:/Fax: 81-76-234-4792

Abstract

Liquid scintillation counting (LSC) method using a radioisotope tracer has several advantages such as simple procedure, high sensitivity and low detection limit, and has been used for the determination Fe concentrations in water samples. Several factors such as nitrate concentration, pH, chelating ligand affect the efficiency of this method in the determination of iron (Fe) in waters. In this study, the effect of nitrate in phytoplankton culture medium on the determination of Fe concentration by LSC method using ^{55}Fe radioisotope tracer was evaluated. The measured Fe concentrations in the medium were lower than its added concentration (1.5 μM) when liquid samples contain nitrate. Fe concentrations decreased exponentially as nitrate concentrations increased up to 2.64 mM, reaching a constant value of 1.31 μM Fe at nitrate concentrations higher than 2.64 mM. A correction factor (f = 1.14) was calculated from the decrease rate of Fe concentrations at different nitrate concentrations in the phytoplankton culture medium. This correction factor can be used to correct the measurement values of Fe concentrations in phytoplankton culture medium obtained from LSC method. Our results showed that up to 94% of the added Fe can be determined by LSC using ^{55}Fe radioisotope tracer. The remaining 6% was probably bound to the walls of the culturing vessel. This method is also applicable for the measurement of Fe size-fractionation in phytoplankton culture medium.

Keywords: Iron, Phytoplankton culture medium, Iron Fractionation, Liquid scintillation counting (LSC) method.

1. Introduction

Iron (Fe) is an essential micronutrient for phytoplankton, which plays important physiological roles in metabolic pathways such as photosynthetic and respiratory electron transport and chlorophyll synthesis [1-3]. Fe also plays a catalytic role in many biochemical reactions as a cofactor of enzymes and proteins involved in chlorophyll synthesis, detoxification of reactive

oxygen species, respiratory and photosynthetic electron transport, and nitrogen assimilation [4]. Fe is the fourth most abundant element in the earth's crust, and one of the important trace metals in seawater [5]. Fe exists primarily as thermodynamically stable trivalent state in seawater [6-8], and its availability in ocean waters is predominately limited by the solubility of Fe(III) [9].

The form of Fe is divided into particulate and dissolved fractions. Most of the inorganic Fe is precipitated as particulate form, such as hydrous oxide at pH 8.0. Dissolved form of Fe is further divided into truly dissolved and colloidal forms. Dissolved Fe can exist in seawater in two oxidation states, Fe(II) and Fe(III), free or complexed with inorganic and organic ligands [8]. It is reported that major fraction of Fe are free inorganic ion, organic complexes, and hydrous Fe oxide [10]. However, the chemical forms of available Fe to phytoplankton are unclear. The measurement of Fe concentration in seawater is therefore important to investigate the Fe uptake mechanism by phytoplankton [11-12]. In general, analytical methods for Fe in seawater needs preconcentration processes because of the low Fe concentration in the ocean [9] compared to its ubiquity and the interference of major salts with sensitive instruments [13-17]. There is also the possibility of contamination during environmental sampling. Furthermore, marine phytoplankton culture medium often contains significant amounts of organic ligand enrichments, which often interfere with Fe measurements using analytical instruments.

The Fe determination methods at trace and ultra-trace levels in seawater are- (1) atomic spectrophotometry (e.g., graphite furnace atomic absorption spectrometry (GF-AAS) or inductively coupled plasma mass spectrometry (ICP-MS)); (2) electrochemical analysis using catalytic cathodic stripping voltammetry; and (3) radio chemical counting of radioactive isotopes ^{55}Fe and ^{59}Fe. Solvent extraction is performed as a preconcentration step in the determination of Fe concentration in seawater by GF-AAS because of insufficient sensitivity of the instrument [12-14, 16]. The concentration of Fe in seawater can be determined at pert per trillion (ppt) level by ICP-MS since the instrumental sensitivity is very high, but the technique needs the preconcentration, desalination process, and the separation of the principal constituent [15-16, 18]. Stripping voltammetry methods

have been developed for sensitive and speciation analysis in the presence of organic ligands, but require pre-processing of acid or microwave treatment for colloids or high Fe concentration [19-22].

In addition to the above analytical techniques, radiochemical analysis (using a radioisotope tracer) can be used for the determination of Fe concentrations in phytoplankton by measuring the ratio of radioactivity. The radiochemical tracer techniques have been used to determine Fe size-fractionation in phytoplankton cultures such as intra- and extracellular Fe [23-24] and Fe(II) production [25]. This method has several advantages such as simple procedure, high sensitivity and low detection limit, and can be useful for the determination of Fe in phytoplankton of laboratory experiments.

^{55}Fe would be a better radioisotope for the stable measurement of Fe concentration by radiochemical analysis during long term experiments because of its longer half-life (2.73 years) compared to that of ^{59}Fe (44.5 days). Previously, chromatographic separation of Fe was performed for the determination of ^{55}Fe in seawater. After separation, ^{55}Fe was determined by counting with a liquid scintillation counter [26]. In this method, isolated ^{55}Fe was determined by a modification of the LSC method. Single label LSC and a quench correction were used for ^{55}Fe determination. In the present study, we examined the effect of nitrate concentration on the measurement of Fe concentration in phytoplankton culture medium by liquid scintillation counting (LSC) method using ^{55}Fe radioisotope tracer. We also investigated the effect of the organic ligands, pH, ^{55}Fe and ^{59}Fe radioisotope tracers, and organic matter released by phytoplankton in the solution on the measurement of Fe concentration and size-fractionation in phytoplankton culture by LSC method. A correction factor was calculated from the decrease rate of Fe concentrations at different nitrate concentrations in the phytoplankton culture medium. This correction factor can be used to correct the measurement values of Fe concentrations in phytoplankton culture medium obtained from LSC method.

2. Materials and Methods

2.1. Reagents

Artificial seawater was prepared by modifying f/2 culture media for phytoplankton according to the method of Lyman and Fleming [27] (Table 1), in which 2-[4-(2-hydroxyethyl)-1-piperazinyle]-ethane sulfonic acid (HEPES; Nacalai Tesque, Japan) was used as a buffer reagent [28]. The standard solution of Fe was prepared by dissolving $FeCl_3 \cdot 6H_2O$ (Wako, Japan) and radioactive isotope ^{55}Fe was prepared by dissolving $^{55}FeCl_3$ (740 MBq, Daiichi Pure Chemicals, Japan) in 1 M HCl. They were diluted to the desired concentrations with E-pure water (EPW, E-pure system, Barnstead, USA), 3% NaCl solution, artificial seawater, and modified f/2 media. Ethylenediamine-N,N,N',N'-tetraaceticacid (EDTA; Dojindo, Japan), diethylenetriamine-N,N,N',N'',N''-tetraaceticacid (DTPA; Dojindo, Japan) and Ethylene-diamine-N,N,N',N'-tetrakis(methylenephosphonicacid) (EDTPO; Dojindo, Japan) were used as chelating ligands. The cocktail for the organic scintillation measurement was prepared by dissolving 3.03 g of 2-(4-tert-Butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole (Dojindo, Japan) in 500 ml of toluene. Other reagents were of analytical reagent grade or better.

Sample solution, EPW, 3% NaCl solution, artificial seawater or modified f/2 culture media were taken in 30 or 250 ml polycarbonate bottles (Nalgene), and the pH was adjusted to either 2, 5, 8, or 11 using 1M HCL or 1M NaOH. Concentrations of Fe, chelating ligands, and nitrate were 1.5 μM (specific activity of 4.11 TBq/mol $FeCl_3$), 0-15 μM, and 0-50 mM, respectively. The temperature of the samples was 20 ˚C during the experiment.

2.2. Measurement of size-fractioned Fe in phytoplankton culture solution

Concentrations of Fe and chelating ligands in the medium were 1.5 μM (containing ^{55}Fe 4.11 TBq/mol $FeCl_3$) and 15 μM, respectively. Phytoplankton *Skeletonena costatum*, which was pre-cultured with Fe-limited media, was inoculated in the experimental culture solution after 48 hours at 20 ˚C. In this study, 0.025, 0.20, and 3.0 μm pore-size polycarbonate filters (Corning, USA) were used to fractionate Fe. The 0.025 μm filter was used to separate colloidal Fe from its particulate

fraction corresponding to 200 kDa of molecular weight, and 0.20 μm filter was used to separate colloidal fraction from its large labile particle fraction. The 3.0 μm filter was used to separate the extracellular Fe that was adsorbed on the surface of phytoplankton cell from its other fractions. For the determination of size fraction, 3 mL aliquots of the sample were filtered through different pore-size filters, and the filter and filtered were used for Fe determination. For the measurement of dissolved Fe, 100 μL of filtered sample was taken in 5 mL of liquid scintillation solution. For the measurement of particulate Fe, the filters were washed with 3 mL of artificial seawater at room temperature, and were soaked in 5 mL of the liquid scintillation solution. Total Fe concentration was determined by adding 100 μL of unfiltered sample solution to 5 mL of liquid scintillation solution. The solution was taken in 20-mL bottles for the measurements by scintillation counting method, and the detection limit of the determination method was 10.8 nM. The concentration of Fe(III) was calculated by the Fe(III)/^{55}Fe(III) ratio in sample solutions.

2.3. Equipments

All radiochemical measurements were carried out on a liquid scintillation counter (LSC-6101, Aloka, Japan). An inductively coupled plasma atomic emission spectrometer (ICP-AES; Optima 3300XL, PerkinElmer, USA) equipped with an ultrasonic nebulizer (U-5000AT, Cetac, USA) was used for the determination of Fe concentrations in standard solution.

3. Results and Discussion

3.1. Effect of chelating ligands, pH and nitrate on the measurement of Fe by LSCM

3.1.1. Effect of chelating ligand

Chelating ligand is generally used to prevent Fe(III) precipitation in phytoplankton culture medium in the laboratory experiments. The effect of chelating ligands such as EDTA, DTPA, and EDTPO on Fe measurement values of scintillation counting method are shown in Figure 1. Whether chelating ligands were added to the solution or not, measured values of Fe concentration in the

modified f/2 culture medium (with or without nitrate) were lower than those in artificial seawater. Regardless of the chelating ligands and control, Fe concentrations were significantly higher in f/2 culture medium without nitrate than in the medium with 0.88 mM nitrate. For example, the measurement values of Fe in EDTA and EDTPO treated f/2 culture medium without nitrate were 1.47±0.03 and 1.48±0.23 μM, while its concentrations were 1.01±0.12 and 1.03±0.22 μM in the medium with 0.88 mM nitrate, respectively. Results indicate that chelating ligands does not affect the measurement of Fe concentration by LSC method while nitrate does affect.

3.1.2. Effect of pH on the measurement of Fe concentration

It was predicted that the measurement of Fe concentration by LSC method might be affected by pH of sample solutions since the chemical form of Fe varies with pH. Fe concentration was measured in EPW, artificial seawater, and modified f/2 culture medium at pH 2, 5, 8, and 11 (Fig. 2). Irrespective of the pH, the measurement values of Fe concentrations in EPW, artificial seawater, and modified f/2 culture medium decreased by 9-10% for the addition of 0.88 mM nitrate in the medium. The lowest measured values of Fe concentrations in EPW, artificial seawater, and f/2 culture medium with nitrate were 1.03±0.09, 1.04±0.00 and 1.01±0.04 μM at pH 2, which differed significantly ($P < 0.05$) with those of the highest values of 1.40±0.06, 1.37±0.05 and 1.30±0.09 at pH 11, respectively. The results indicate that pH also affect the measurement of Fe concentration by the LSC method.

3.1.3. Effect of nitrate on the measurement of size-fractioned Fe in the medium

To investigate the effect of nitrate on the measurement of Fe concentrations of different size-fractions (e.g., particulate, colloidal, and soluble fractions) by the LSC method, the culture media were filtered through 0.20 μm filters and the Fe concentrations were measured in both the filtered and unfiltered medium. Irrespective of addition of nitrate in the medium, Fe in the filtered samples of EPW and artificial seawater was found mostly (> 90% of the total Fe) of < 0.20 μm fraction,

while about half of the Fe in modified f/2 culture medium was found to be of > 0.20 μm fraction (Fig. 3). In addition, Fe concentrations in the modified f/2 medium without nitrate were 1.31±0.05 and 0.58±0.05 μM, which were decreased in the medium with nitrate to 1.20±0.07 and 0.52±0.02 μM for unfiltered and filtered samples, respectively (Fig. 3C). On the other hand, the measurement values of Fe concentrations of < 0.20 μm fraction in the filtered were almost same; 0.33±0.04 and 0.34±0.04 μM in the medium without and with 0.88 mM nitrate, respectively (Fig. 3C). Results indicate that the addition of nitrate in the medium apparently decreased the concentration of Fe in the samples, which would be because nitrate interfered with scintillation counting of χ-ray for ^{55}Fe.

In the LSC method, the measurement efficiency of Fe concentration may be decreased for quenching effect that may occurs from impurities, additives, and radioactive material in liquid scintillation counter [29]. Several correction methods such as internal standard method, sample channel ratio method, and external standard were used to correct measurement efficiency. However, these methods are applicable to get reliable measurement values of Fe concentrations if the quenching is stable or the number of samples is few. Other correction method may be required for the measurement of Fe concentration by this method since the quenching effect of nitrate depends on the nitrate concentrations in the samples.

3.1.4. Influence of nitrate on the measurement of Fe concentrations using ^{55}Fe and ^{59}Fe

In the present study, the effect of radioisotope tracer (^{55}Fe and ^{59}Fe) on the measurement of Fe concentrations in phytoplankton culture medium by LSC method was investigated. When ^{55}Fe radioisotope tracer was used, the measurement values of Fe concentrations in EPW, artificial seawater and modified f/2 medium without nitrate were 1.54±0.04, 1.50±0.14, and 1.40±0.08 μM that decreased to 1.39±0.11, 1.34±0.14, and 1.25±0.07 μM, respectively, for the addition of 0.88 mM nitrate, and the correction rate differed by 10% for the addition of nitrate (Fig. 4A). On the other hand, measurement values of Fe concentrations in EPW, artificial seawater and modified f/2 medium were almost identical when ^{59}Fe was used as radioisotope tracer, and the values did not

depend on nitrate in the medium (the Fe concentrations were 1.49±0.06, 1.38±0.08, and 1.39±0.13 µM in EPW, artificial seawater and modified f/2 media without nitrate, and 1.45±0.09, 1.37±0.04, and 1.42±0.11 µM in the medium with 0.88 mM nitrate, respectively).

In addition, when ^{59}Fe radioisotope tracer was used for the measurement of Fe concentrations by γ-counting method, the correction rates of the measurement values did not differ for the addition of nitrate in the medium (Fig. 4B). The measurement values were 96864±87, 98680±94, 98628±182 cpm for EPW, artificial seawater and modified f/2 culture medium without nitrate and 98249±103, 98801±91, 97784±149 cpm for medium with 0.88 mM nitrate, respectively. It is suggested that nitrate cannot affect the measurement of Fe concentration when ^{59}Fe radioisotope tracer is used in LSC method because the frequency of excitation of the scintillator is related to the half-life of Fe radio-isotopes.

3.2. Correction factor (*f*) for the measurement of Fe concentrations

The effect of nitrate concentration on the measurement of Fe concentration in phytoplankton culture medium based on LSC measurements using ^{55}Fe was investigated. The measurement values of Fe concentrations decreased exponentially up to 2.64 mM nitrate in the medium and then remain steady (about 1.31 µM) up to 44 mM nitrate concentration (Fig. 5A). Using these data, the decrease rate of the measurement values of Fe concentrations by LSC method for the addition of nitrate in the medium was calculated by the following formula:

$$Decrease\ rate\ (\%) = \left\{\frac{(Fe^{I} - Fe^{II})}{Fe^{II}} \times 100\right\} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (I)$$

Where, *Fe' and Fe"* are Fe concentrations in culture medium without and with nitrate, respectively. The results showed that Fe concentrations increased with increasing nitrate concentrations up to 2.64 mM, and then remain constant up to 44 mM nitrate concentrations (Fig. 5B). Using the Fe concentrations in the medium of each nitrate treatment, the corrected concentrations of Fe was calculated by the following formula:

$$Corrected\ concentration\ of\ Fe\ [Fe]_c = f \times [Fe]_m \ldots\ldots\ldots\ldots\ldots\ldots (II)$$

Where, $[Fe]_c$ = the corrected concentration of Fe; $[Fe]_m$ = measured value of Fe concentration at each nitrate treatment. The correction factor (f = 1.14) that was calculated from the decrease rate of Fe concentrations at different nitrate concentrations. This correction factor can be applied at nitrate concentrations > 2.64 mM in the medium. However, we recommend 10 mM nitrate as matrix modifier.

3.3. The measurement of Fe concentration in phytoplankton culture medium

3.3.1. Effect of organic matter released by phytoplankton

The effect of organic matter that may be released by phytoplankton during culture on the measurement of Fe concentrations in the modified f/2 culture medium by LSC method was investigated. Fe concentrations were measured in unfiltered and filtered (through 3.0 µm filter) medium at logarithmic growth phase of phytoplankton culture. Fe concentrations were 1.48±0.03 and 1.35±0.02 µM in unfiltered medium without and with 0.88 mM nitrate, respectively, and its concentrations in filtered medium did not differ significantly for the addition of nitrate (Fig. 6). The corrected concentrations of Fe were 1.51±0.03 and 1.45±0.03 µM in the unfiltered medium without and with 0.88 mM nitrate, respectively. These values correspond to the approximate concentration of Fe added to prepare the medium. The result indicates that the organic matter released by the phytoplankton do not affect the measurement values of Fe concentrations in the phytoplankton culture medium by LSC method, and thus, this method can be used for the determination of Fe concentration in phytoplankton.

3.3.2. Measurement of size-fractioned Fe concentrations in the phytoplankton culture

Fe in the phytoplankton culture was speciated/fractionated into truly dissolved (< 0.025 µm), colloidal (0.025-0.20 µm), large labile particle (0.20-3.0 µm), and particulate (> 3.0 µm) fractions using LSC method (Fig. 7). Fe concentrations in the unfiltered modified f/2 culture medium

decreased from 1.46 μM to about 1.11 μM within 12 h of incubation and remain constant up to 96 h, which was significantly lower than added Fe (1.5 μM) in the phytoplankton culture medium. It might be due to the adsorption of Fe on the bottle wall and/or precipitation as hydrous Fe oxides [30]. The truly dissolved fraction (< 0.025 μm) of the Fe was almost constant (about 0.77 μM) throughout the experiment. On the other hand, Fe concentration of large labile particulate fraction (0.20-3.0 μm) increased to 0.39 μM from its initial concentration (0.10 μM) within 6 h of phytoplankton inoculation in the culture medium and then decreased gradually. The concentration of colloidal fractions (0.025-0.20 μm) of Fe decreased gradually with the increase of incubation time, while the concentration of particulate fraction (> 3.0 μm) increased gradually after the inoculation of phytoplankton in the culture medium and reached to 0.09 μM after 96 h of incubation. The results indicate that the colloidal fraction of the Fe was absorbed on the phytoplankton cell surface and/or was partially taken up in the phytoplankton cells. The decrease of large labile particulate Fe fraction (0.2-3.0 μm) was most likely transferred to the colloidal Fe fraction.

In addition, Fe concentrations in unfiltered culture medium and sum of other fractions (dissolved + colloidal + large labile particle + particulate fractions), measured by LSC method at the time of phytoplankton inoculation, were 1.46±0.03 and 1.35±0.09 μM, respectively, which is about 94% of its added concentration in the unfiltered medium. Therefore, the LSC method can be used in the size-fractionation analysis of Fe.

Conclusions

Nitrate is an important nutrient for marine phytoplankton culture medium that affects the determination of Fe concentrations in the medium by LSC method using ^{55}Fe radioisotope tracer. Nitrate decreases the measurement values of Fe concentration when ^{55}Fe radioisotope tracer is used in LSC method. One of the approaches to the solution of this problem would be the addition of nitrate to all the liquid samples as well as the quench curve samples as a matrix modifier. The counting efficiency of ^{55}Fe by the LSC method is constant in the range of 2.64-44.0 mM nitrate,

where it is possible to yield reproducible measurements and linear behavior for Fe determination. The decreased measurement values can also be corrected using a correction factor (f = 1.14) that is calculated from the decrease rate of Fe concentrations in the medium at different nitrate concentrations. This correction factor is more suitable at 10 mM nitrate concentration in the medium. Since nitrate does not affect the measurement values of Fe concentrations when ^{59}Fe radioisotope tracer is used in the LSC method, the use of ^{59}Fe radioisotope tracer instead of ^{55}Fe can be a solution to the problem of decreased measurement values. In addition, the LSC method can also be used effectively to determine Fe fractions in the phytoplankton culture medium.

Acknowledgements

This research was partly supported by a Grant-in-Aid for Scientific Research (18510071) from Japan Society for the Promotion of Science (JSPS) and the Steel Industry Foundation for the Advancement of Environmental Protection Technology, Japan. The authors also wish to thank the University of Technology, Sydney (UTS), Australia, for supporting the research. We also would like to thank Dr. Anne Colville, School of Environmental Sciences, University of Technology Sydney (UTS), Australia, for reviewing the manuscript and making a number of helpful suggestions prior to submission.

References

[1] Geider R and Roche J (1994) Photosynth Res 39: 275-301.

[2] Sunda WG, Swift DG and Huntsman SA (1991) Nature 351: 55-57.

[3] Sunda WG and Huntsman SA (1997) Nature 390: 389-392.

[4] Maldonado MT and Price NM (2000) Limnol Oceanogr: 814-826.

[5] Taylor SR (1964) Geochim Cosmochim Acta 28: 1273-1285.

[6] Morel FMM and Hering JG, Principles and Applications of Aquatic Chemistry, Wiley-Interscience, New York, (1993) p.

[7] Kuma K, Nishioka J and Matsunaga K (1996) Limnol Oceanogr 41: 396-407.

[8] Liu X and Millero FJ (2002) Mar Chem 77: 43-54.

[9] Johnson KS, Gordon RM and Coale KH (1997) Mar Chem 57: 137-161.

[10] Lewin J and Chen C (1973) Limnol Oceanogr 18: 590-596.

[11] Nishioka J and Takeda S (2000) Mar Biol 137: 231-238.

[12] Kuma K, Katsumoto A, Shiga N, Sawabe T and Matsunaga K (2000) Mar Chem 71: 111-123.

[13] King JN and Fritz JS (1985) Anal Chem 57: 1016-1020.

[14] Van Geen A and Boyle E (1990) Anal Chem 62: 1705-1709.

[15] Sohrin Y, Iwamoto S-i, Akiyama S, Fujita T, Kugii T, Obata H, Nakayama E, Goda S, Fujishima Y, Hasegawa H, Ueda K and Matsui M (1998) Anal Chim Acta 363: 11-19.

[16] Wells ML and Bruland KW (1998) Mar Chem 63: 145-153.

[17] Gobler CJ, Donat JR, Consolvo Iii JA and Sañudo-Wilhelmy SA (2002) Mar Chem 77: 71-89.

[18] Akatsuka K, McLaren JW, Lam JW and Berman SS (1992) J Anal At Spectrom 7: 889-894.

[19] van den Berg CMG (1995) Mar Chem 50: 139-157.

[20] Wu J and Luther GW (1995) Mar Chem 50: 159-177.

[21] Rue EL and Bruland KW (1997) Limnol Oceanogr 42: 901-910.

[22] Bruland KW, Rue EL and Smith GJ (2001) Limnol Oceanogr 46: 1661-1674.

[23] Hudson RJM and Morel FMM (1989) Limnol Oceanogr 34: 1113-1120.

[24] Hudson RJM and Morel FMM (1990) Limnol Oceanogr 35: 1002-1020.

[25] Shaked Y, Kustka AB, Morel FMM and Erel Y (2004) Limnol Oceanog: Methods 2: 137-145.

[26] Grahek Ž and Rožmarić MM (2005) J Radioanal Nucl Chem 267: 131-137.

[27] Lyman J and Fleming RH (1940) Journal of Marine Research 3: 134-146.

[28] Watanabe MM, Kawachi M, Hiroki M and Kasai F, NIES Collection List of Strains: Microalgae and Protozoa, National Institute for Environmental Studies, Tsukuba, Japan, (2000) p. 159.

[29] Malonda AG, Free Parameter Models in Liquid Scintillation Counting, CIEMAT, Madrid, (1999) p. 416.

[30] Kuma K, Nakabayashi S, Suzuki Y and Matsunaga K (1992) Mar Chem 38: 133-143.

Table 1: The composition of modified f/2 phytoplankton culture media prepared with artificial seawater

Component	Total concentration (mol L^{-1})
$NaNO_3$	88.2×10^{-5}
$NaH_2PO_4 \cdot 2H_2O$	38.5×10^{-6}
$Na_2SiO_3 \cdot 9H_2O$	35.2×10^{-6}
$CoSO_4 \cdot 7H_2O$	42.7×10^{-9}
$ZnSO_4 \cdot 7H_2O$	73.0×10^{-9}
$MnCl_2 \cdot 4H_2O$	90.9×10^{-8}
$CuSO_4 \cdot 5H_2O$	28.0×10^{-9}
$Na_2MoO_4 \cdot 2H_2O$	28.9×10^{-9}
SeO_2	10.0×10^{-9}
Vitamin B_{12}	36.9×10^{-11}
Biotin	20.5×10^{-10}
Thiamine HCl	29.7×10^{-8}
HEPES	50.4×10^{-7}

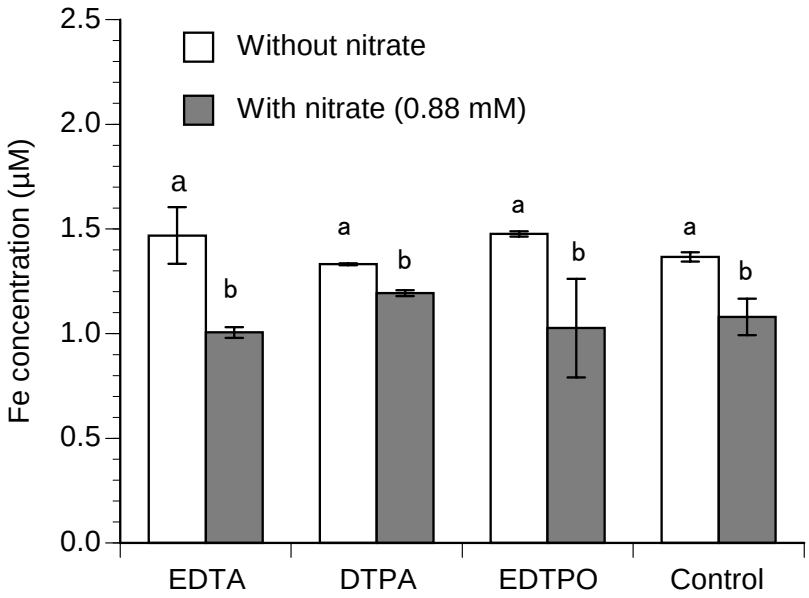

Fig. 1: Effect of chelating ligands on the measurement values of Fe by scintillation counting method with or wihtout nitrate. In control, the f/2 artificial seawater did not contain chelating lignad. Added Fe concentration in the medium was 1.5 µM (specific activity of 4.11 TBq/mol $FeCl_3$). Different letter in indicates significant difference between the treatments ($P < 0.05$). Values are mean ± SD ($n = 3$).

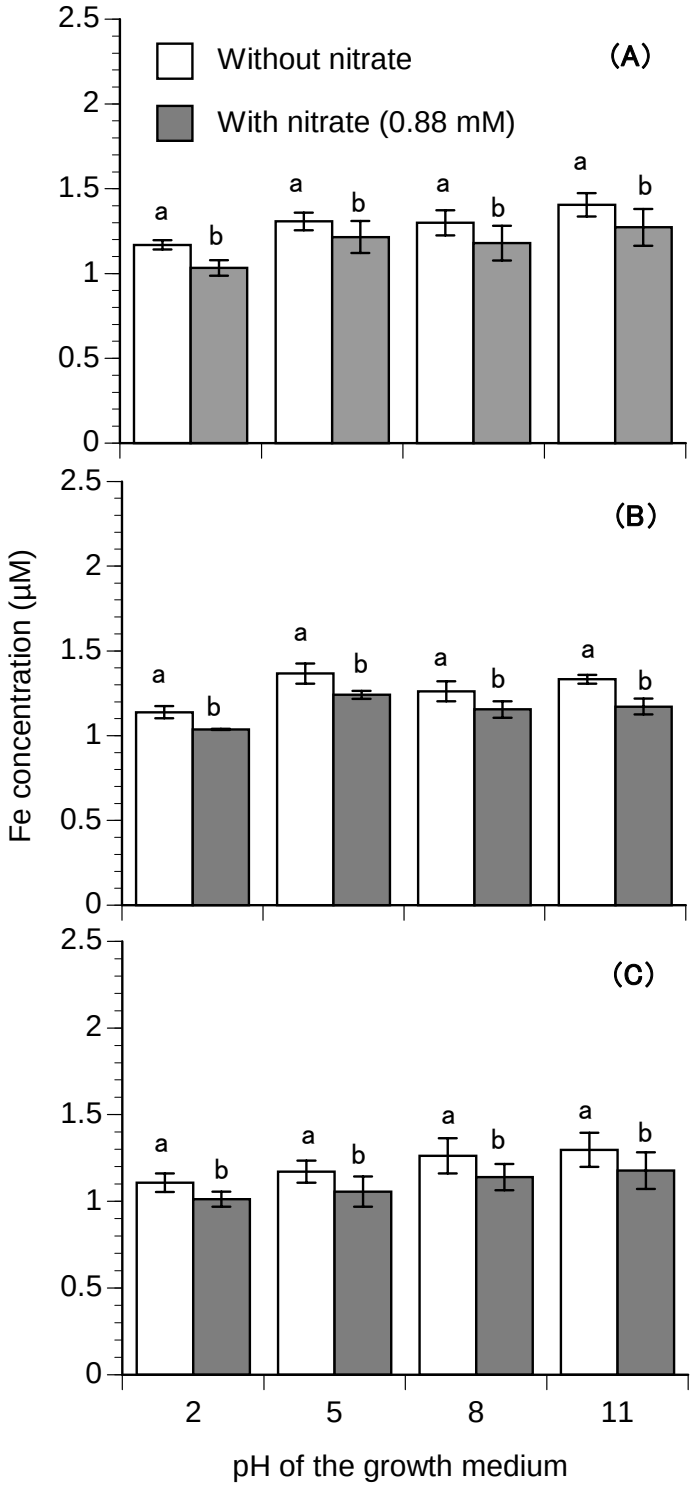

Fig. 2: Effect of pH on the measurement values of Fe by liquid scintillation counting method with or wihtout nitrate. Added Fe concentration in the medium was 1.5 µM containing ^{55}Fe 4.11 TBq mol^{-1} $FeCl_3$. E-pure water **(A)**; artificial seawater **(B)**; f/2 culture medium **(C)**. Different letter in indicates significant difference between the treatments ($P < 0.05$). Values are mean ± SD ($n = 3$).

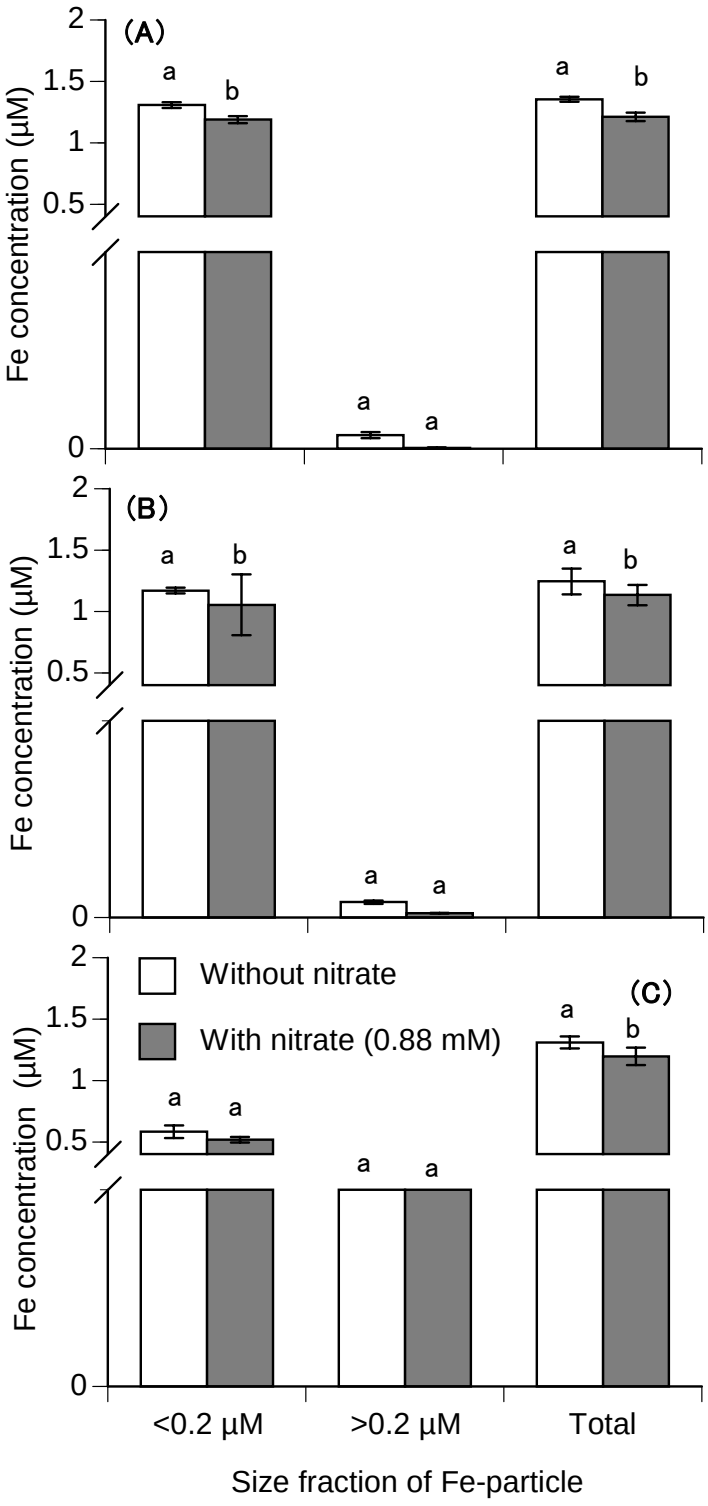

Fig. 3: Effect of nitrate on the determination of size-fraction Fe concentrations by liquid scintillation counting method. Added Fe concentration in the medium was 1.5 µM containing ^{55}Fe 4.11 TBq mol^{-1} $FeCl_3$. E-pure water **(A)**; artificial seawater **(B)**; f/2 culture medium **(C)**. Different letter in indicates significant difference between the treatments ($P < 0.05$). Values are mean ± SD ($n = 3$).

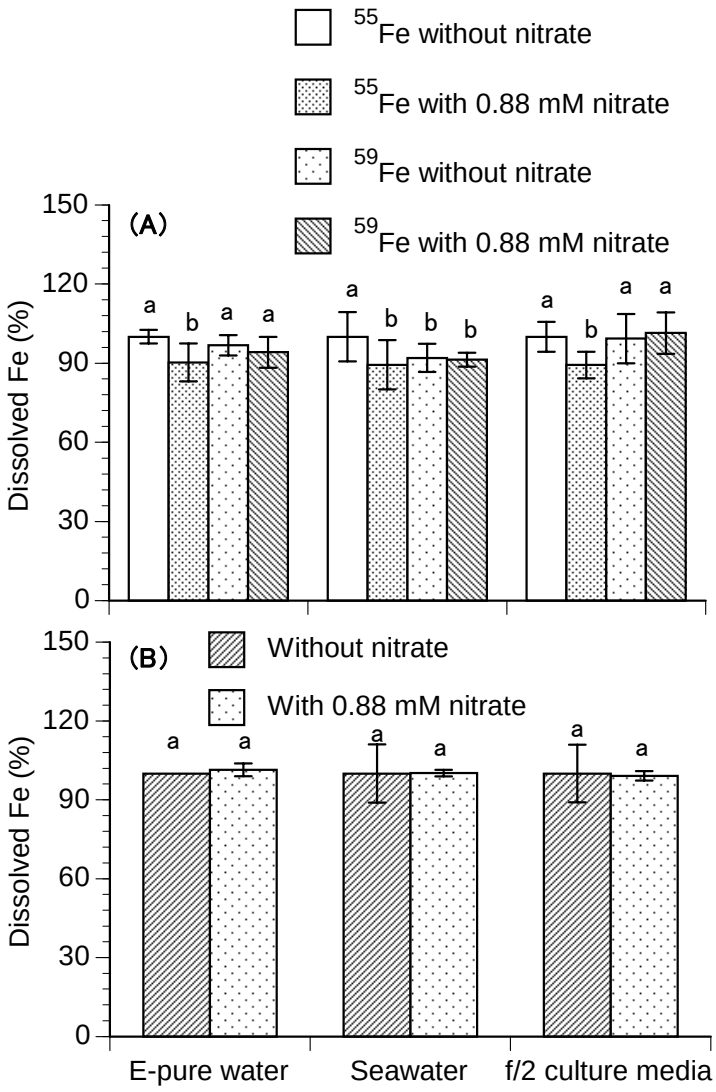

Fig. 4: Effect of nitrate on the measurement of dissolved Fe concentrations in phytoplankton culture media (E-pure water, artificial seawater and modified f/2 medium) by liquid scintillation counting method using ^{55}Fe and ^{59}Fe radioisotope tracers **(A)** and by γ-counter method **(B)**. Different letter in indicates significant difference between the treatments ($P < 0.05$). Values are mean ± SD ($n = 3$).

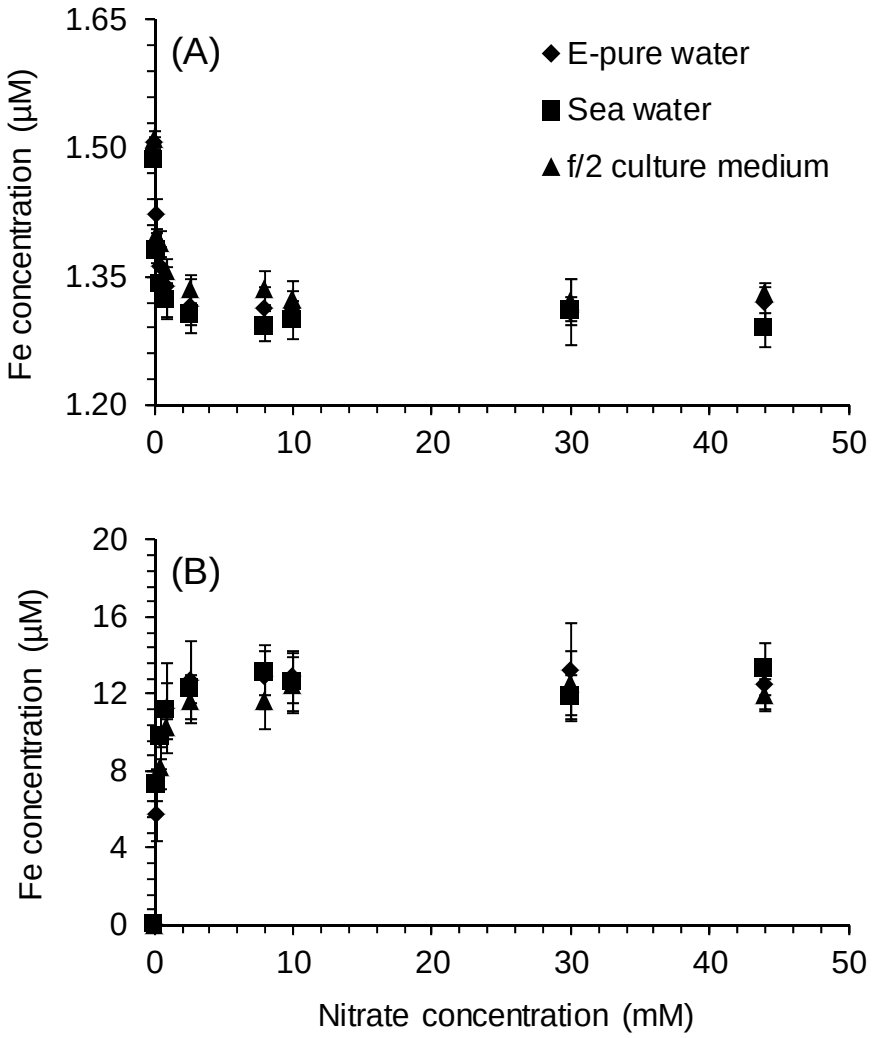

Fig. 5: Effect of nitrate concentrations on the measurement of Fe concentrations in phytoplankton culture medium based on LSC measurements using ^{55}Fe. Values are mean ± SD (n = 3).

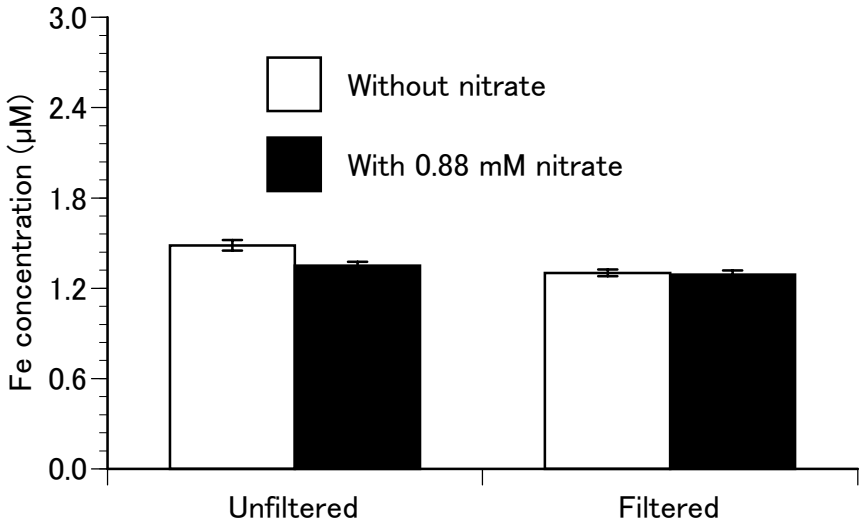

Fig. 6: Effect of organic matter, released by phytoplankton during culture, on the measurement of Fe concentrations in modified f/2 culture medium by liquid scintillation counting method. The medium was filtered with 0.20 µm polycarbonate filter. Values are mean ± SD (*n* = 3).

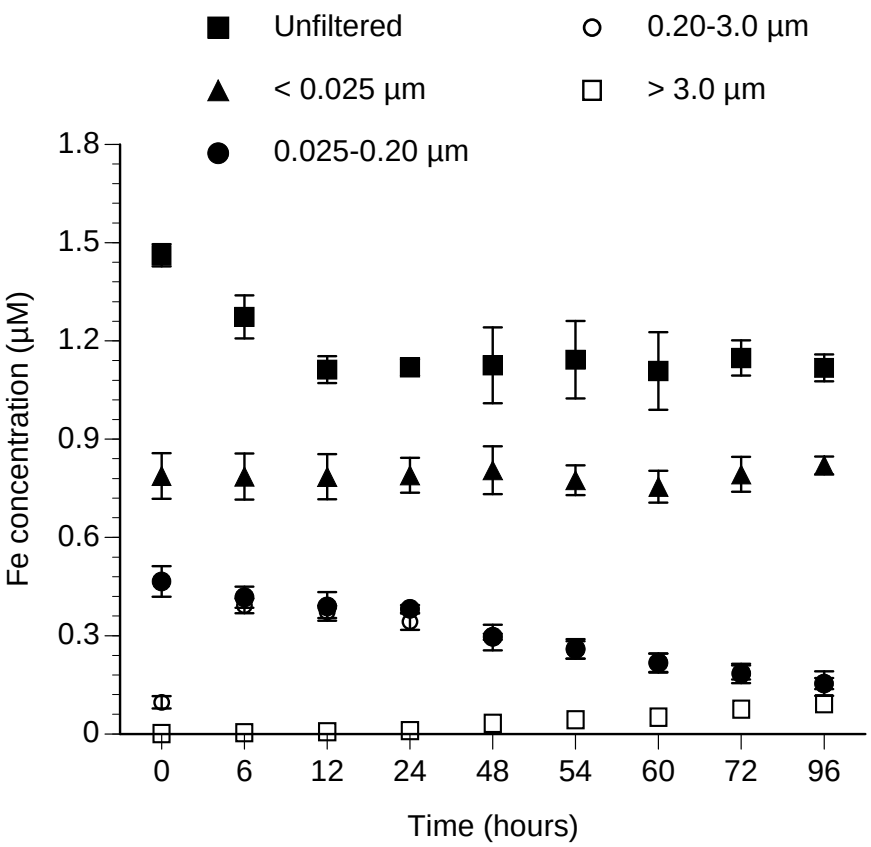

Fig. 7: Size-fraction measurement of Fe in the modified f/2 culture medium during phytoplankton culture by liquid scintillation counting method.