Determination of Iron in Phytoplankton Cultures by Radiochemical Analysis with $^{55}$Fe

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Radiochemical analysis using iron tracer, $^{55}$Fe, with liquid scintillation counting is a simple, sensitive and reliable method for determination of low-level iron, and is useful for iron speciation in laboratory culture experiments of marine phytoplankton. In this paper, we studied interferences from the components of the culture media, established the analytical conditions, and measured iron distribution including dissolved, particle, intra- and extracellular iron in marine phytoplankton cultures. The results from iron uptake experiments showed that bioavailability of iron to phytoplankton was related to the iron speciation in the presence of the chelating ligands.

Key Words: iron, radiochemical analysis, phytoplankton culture, trace element, quenching

1. Introduction

Chemical speciation of iron around marine phytoplankton has received considerable attention since iron may regulate productivity in large regions of the ocean$^{12}$. Although a great deal of effort has been made on iron limitation, what remains to be resolved is to understand chemical forms of iron in seawater and their bioavailability to phytoplankton. Analytical methods for iron speciation in seawater need sensitivity, reproducibility and stability enough to determine low concentrations of iron in the presence of major salts and organic substances which interfere with iron measurements using analytical instruments. In this respect, radiochemical analysis using a radioisotope tracer is effective for the laboratory cultures of marine phytoplankton, and also has advantages of a simple procedure and low contamination. Two radioisotopes, $^{54}$Fe and $^{55}$Fe, are often used for an iron tracer.

In this study, we developed an analytical method using a radioisotope tracer $^{55}$Fe for iron speciation in seawater media. We also present the distribution of iron, including dissolved, particulate, intracellular and extracellular concentrations in the phytoplankton cultures.

2. Materials and Methods

2.1 Materials and Reagents

All solutions were prepared with purified water (EPW) using an E-pure system (Barnstead, USA) and artificial seawater was prepared according to the method of Lyman and Fleming$^{3}$. A stock solution of Fe(III) was prepared by dissolving FeCl$_3$·6H$_2$O (Nacalai Tesque, Kyoto, Japan) in 1M HCl (TAMAPURE-AA-100, Tama Chemicals, Tokyo, Japan) and standardized by using ICP-AES. A stock solution of $^{55}$Fe(III) was prepared by dissolving $^{55}$FeCl$_3$ solution (PerkinElmer Life & Analytical Sciences, 37 MBq) in 1M HCl (TAMAPURE-AA-100). Stock solutions (10$^{-2}$ M) of chelating ligands were prepared by dissolving the corresponding compounds (Dojindo Molecular Technologies; Fig. 1) in 0.1 M sodium hydroxide. They were diluted to the desired concentrations. Other reagents were of analytical reagent grade or better.

![Chemical structures of chelating ligands](image)

2.2 Apparatus

Radiochemical measurements for $^{55}$Fe 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 determination of iron concentrations. Phytoplankton growth was measured by fluorometrical measurements on a fluorometer (TD-700, Turner Designs).

2.3 Culture Experiments
An axenic culture of *Prymnesium parvum* (Haptophyceae) was used. Before the experiments, the cultures were maintained in the modified f/2 media 1 (Table 1) reducing the concentrations of Fe(III) to 0.1 μM and omitting chelating ligands until cells were at an exponential phase of growth. Experimental cultures were grown at 20 °C under a 12:12 h L/D photoperiod at a light intensity of 150 μmol m⁻² s⁻¹ provided by cool white fluorescent lights.

Twenty-milliliter aliquots of the modified f/2 medium were pipetted into 30-ml capacity acid-washed polycarbonate bottles. After sterilization by autoclaving, concentrations of Fe(III) and chelating ligands were adjusted to 1.5 μM and 15 μM, respectively. The media were inoculated with acclimated exponential phase cells of phytoplankton which resulted in 20 cells ml⁻¹ for a period of 24 hours. Samples were taken from each bottle in a clean boom.

<table>
<thead>
<tr>
<th>Component</th>
<th>Total concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>8.8 x 10⁻⁴</td>
</tr>
<tr>
<td>Na₂HPO₄·2H₂O</td>
<td>3.9 x 10⁻⁵</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>3.7 x 10⁻¹⁰</td>
</tr>
<tr>
<td>Biotin</td>
<td>2.1 x 10⁻⁹</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>3.0 x 10⁻⁷</td>
</tr>
<tr>
<td>Na₂SiO₃·9H₂O</td>
<td>3.5 x 10⁻⁵</td>
</tr>
<tr>
<td>f/2 metal CoSO₄·7H₂O</td>
<td>4.3 x 10⁻⁸</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>7.3 x 10⁻⁸</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>9.1 x 10⁻⁷</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>2.8 x 10⁻⁸</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>2.9 x 10⁻⁸</td>
</tr>
<tr>
<td>Na₂SeO₃</td>
<td>1.0 x 10⁻⁸</td>
</tr>
<tr>
<td>HEPES</td>
<td>5.0 x 10⁻³</td>
</tr>
</tbody>
</table>

Table 1 Composition of modified f/2 media

2.4 Size Fractionation of Iron in Culture Media
Conventional filtration with membrane filters of 0.025, 0.2 and 3-μm pore size was used to separate four iron fractions: <0.025 μm, 0.025-0.2 μm, 0.2-3.0 μm and >3.0 μm. To measure dissolved Fe(III) in liquid samples, 100-μl aliquots of the samples were added to 5 ml of liquid scintillation solutions (2-(4-tert-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiaze 0.53 g / toluene 500 ml) in 20 ml vials. A radiochemical activity of ⁵⁵Fe was measured using the tritium mode of the liquid scintillation counter. The concentration of Fe(III) was calculated by the Fe(III)/⁵⁵Fe(III) ratio in sample solutions.

2.5 Chemical Speciation of Iron in Phytoplankton Cells
Extra- and intracellular fractions of iron in phytoplankton cells were determined according to the method described by Hudson and Morel [5]. Two 5-ml aliquots were collected from a culture medium, and filtered through 3.0-μm pore size filters (Nuclepore). For intracellular iron, one filter was successively rinsed with 5 ml of the artificial seawater, 5 ml of 0.047 M Ti(III)/citrate/EDTA solution and 5 ml of the artificial seawater on a filter holder. For total iron (corresponding to intra and extracellular iron), another filter was rinsed with 5 ml of the artificial seawater. Both of the filters, on which ⁵⁵Fe retained as a tracer, were directly added to 5 ml of the liquid scintillation solutions, and the concentrations of iron were determined in a similar manner as above.

3. Results and Discussion
3.1 Determination of Iron using a Radioisotope Tracer ⁵⁵Fe
The concentrations of dissolved iron in culture media and particulate iron collected on filters were determined by using a radioisotope tracer ⁵⁵Fe with liquid scintillation counting. Although the radioanalysis of ⁵⁵Fe is applicable to the observation of iron using particulate samples on filters, evaluation of quenching effect is required to determine iron concentrations in aqueous samples. Table 2 shows influences of the major components in the modified f/2 media on scintillation counting of ⁵⁵Fe. It is found that the loss of the counting in the medium without nitrate is negligible, while the other media depress the ⁵⁵Fe counting. The results suggest that nitrate reduces the counting efficiency of ⁵⁵Fe.

<table>
<thead>
<tr>
<th>Eliminated components from f/2 media</th>
<th>NO₃⁻</th>
<th>PO₄³⁻</th>
<th>SiO₂⁻</th>
<th>⁵⁵Fe metals</th>
<th>SeO₂⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counting efficiency</td>
<td>99.3</td>
<td>88.7</td>
<td>93.4</td>
<td>90.3</td>
<td>92.1</td>
</tr>
</tbody>
</table>

Table 2 Influence of the components in the modified f/2 media on determination of ⁵⁵Fe with liquid scintillation.

Generally, aqueous samples containing ⁵⁵Fe species and hydrophobic liquid scintillators are mixed into a homogenous and gelatinous composition with emulsifiers in order to increase excitation of the scintillators. However, this conventional method also increases the interference of nitrate quenching. Okumura et al. provides a detailed discussion of interference that influences the measurement of ⁵⁵Fe in seawater media. In this study, the problem was solved by using a liquid scintillator without
emulsifiers, which made a hydrophobic layer with scintillators and a aqueous layer containing nitrate in scintillation vials. The interference of nitrate was saturated over 1.8 mM nitrate, because the quenching is limited to the interface of the layers. In all measurements, 4.4 mM nitrate was added to sample solutions as a matrix modifier. The counting efficiency of $^{55}$Fe is almost constant, 90.9±2.7% (n=5), and the detection limit calculated by 3 times the standard deviation of the blank is 11 nM for 2.4 x 10^{11} mol/Bq of the Fe(III)/$^{55}$Fe(III) solutions.

3.2 Effect of Phytoplankton Cells on Iron Speciation in Culture Medium

Change in size fraction of iron was observed in the culture medium of *P. parvum* (Fig. 2). Before addition of the cells, the main fractions of iron were <0.025 μm and 0.20-3.0 μm. Immediately after Fe(III) and EDTA were adjusted to 1.5 μM and 15 μM at time = 0, the fraction of 0.20-3.0 μm increased up to 0.5 μM. At 12-48 h, iron distribution was relatively steady; the iron concentrations of <0.025 μm, 0.025-0.20 μm, 0.20-3.0 μm and >3 μm were 0.92±0.01 μM, 0.05±0.02 μM, 0.46±0.03 μM, and 0.04±0.02 μM, respectively. When the cells of *P. parvum* were added at 49 h, the >3.0 μm fraction markedly increased instead of the 0.20-3.0 μm fraction. The results suggest that the cells of phytoplankton adsorb the 0.20-3.0 μm fraction of iron preferentially. Nishioka and Takeda reported the similar decrease of small colloidal fraction (200 kDa -0.2 μm) of iron in natural seawater\(^7\). It is considered that the distribution of colloidal fraction depends on the concentrations of iron and other components in the media.

![Fig. 2](image)

**Fig. 2** Size fraction of iron in the culture of *P. parvum*. Total iron (•), >0.025 μm (□), >0.20 μm (O) and >3.0 μm (Δ). Initial concentration: Fe(III); 1.5 μM of truly dissolved form (<0.025 fraction), EDTA; 15 μM, cells of *P. parvum*; 2x10^4 cells ml^{-1}. At 49 hours, the cells of *P. parvum* were added in the medium which resulted in 2x10^4 cells ml^{-1}.

3.3 Iron Speciation in Phytoplankton Cells

Fig. 3 shows the formation of intra- and extracellular iron in the cells of *P. parvum* after the cells were added to the modified f/2 medium containing 1.5 μM Fe(III) and 15 μM EDTA. Before the experiments, the cells were maintained in the modified f/2 media to reduce the concentrations of Fe(III) to 0.1 μM and to omit chelating ligands until the cells reached an exponential phase of growth.

In Fig. 3, a fraction of extracellular iron is given as the difference between total and intracellular iron. Extracellular iron rapidly increased to 0.75 pmol cell\(^{-1}\) at 2 h, and gradually increased to the end of the experiment. Intracellular iron also showed a similar behavior although the concentrations were 11-15% of extracellular iron.

![Fig. 3](image)

**Fig. 3** Particulate iron formation (>3.0 μm) in the modified f/2 media containing *P. parvum* cells. Initial condition: Fe(III); 1.5 μM of truly dissolved form (<0.025 fraction), EDTA; 15 μM, cells of *P. parvum*; 2x10^4 cells ml^{-1}. Total iron (○; a sum of intra- and extracellular iron) and intracellular iron (△).

3.4 Iron Speciation in the Modified f/2 Media Containing Artificial Chelating Ligands

Recent studies have shown that most of iron in seawater is bound to natural organic ligands\(^1\). In laboratory experiments, an artificial chelator, such as EDTA and nitritriacetic acid (NTA), was commonly used in order to obtain a constant and controlled supply of iron. In the presence of chelating ligands, the bioavailability of iron and its chemical speciation are highly influenced each other\(^2\).

Fig. 4 shows the effects of chelating ligands on the growth of *P. parvum* in the modified f/2 media containing 1.5 μM of Fe(III). The growth curves of *P. parvum* depended on the species and concentrations of the chelating ligands in the media. When the culture media contained 15 μM and 150 μM of EDTA and EDTA-OH as chelating ligands, the growth curves were almost equal to those of control without any chelating ligands. On the other hand, addition of DTPA and EDTPO depressed the cell growth as the concentrations increased. The depressed effect of the chelating ligands was also reported in other phytoplankton cultures\(^3\). Little difference in the growth rates was observed in the media containing 6.0 μM of iron.
Fig. 4  Effects of artificial chelating ligands on the growth of P. parvum. EDTA and EDTA-OH (□), DTPA (○), EDTPO (△) and control (●). Initially, the media contained 1.5 μM of Fe(III) without chelating ligand. At 5 hours, the chelating ligands were added in the media which resulted in (a) 15 μM and (b) 150 μM.

The formation of intra- and extracellular iron of P. parvum in the presence of the chelating ligands is shown in Fig. 5. The concentrations of Fe(III) and the ligands were the same as the growth experiments in Fig. 4 (a). Iron uptake of the cells in the presence of EDTPO was unstable and less than 10^3 pmol cell^(-1), while those of EDTA, EDTA-OH and DTPA increased during the experiments. The amounts of iron uptake within 48 hours were as follows;

EDTA > EDTA-OH > DTPA > EDTPO  

The behavior of iron uptake was consistent with the depressed effect on the cell growth shown in Fig. 4. These results indicated that the availability of iron to phytoplankton was responsible for the depressed effect of chelating ligands. Complexation of iron with chelating ligands would interfere with iron transport reactions on the cells. It is also reported that the iron availability is related to the ratios of iron to chelating ligands in the media and the abilities of the ligands to complex Fe(III) and other ions.\(^{59}\).

4. Conclusion

The radiochemical analysis using \(^{55}\)Fe as a tracer and liquid scintillation is convenient because of simplicity and reliability. From the viewpoint of stable measurements, \(^{55}\)Fe (half-life of 2.73 years) is a more useful radioisotope of iron than \(^{59}\)Fe (44.5 days). We estimated the interference of nitrate on the measurement of \(^{55}\)Fe, and showed the detailed behavior of iron species in marine phytoplankton cultures containing chelating ligands. The findings are applicable for accurate determination of iron concentrations in phytoplankton cultures and will make a contribution to studies to elucidate the biogeochemistry of iron in the environment.

Fig. 5  Effect of chelating ligands on particulate iron formation (>3.0 μm) in the modified f/2 media containing P. parvum cells. Initial condition: Fe(III); 1.5 μM, chelating ligands; 15 μM, cells of P. parvum; 2x10^5 cells ml^-1. Total iron (●): a sum of intra- and extracellular iron and intracellular iron (○).

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References