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# Determination of ferrous and ferric iron in aqueous biological solutions

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ABSTRACT

A solvent extraction method was employed to determine ferrous and ferric iron in aqueous samples. $Fe^{3+}$ is selectively extracted into the organic phase (*n*-heptane) using HDEHP (bis(2-ethylhexyl) hydrogen phosphate) and is then stripped using a strong acid. After separation, both oxidation states and the total iron content were determined directly by ICP-MS analysis. This extraction method was refined to allow determination of both iron oxidation states in the presence of strong complexing ligands, such as citrate, NTA and EDTA. The accuracy of the method was verified by crosschecking using a refinement of the ferrozine assay. Presented results demonstrate the ability of the extraction method to work in a microbiological system in the presence of strong chelating agents following the bioreduction of $Fe^{3+}$ by the *Shewanella alga BrY*. Based on the results we report, a robust approach was defined to separately analyze $Fe^{3+}$ and $Fe^{2+}$ under a wide range of potential scenarios in subsurface environments where radionuclide/metal contamination may coexist with strongly complexing organic contaminants.

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## 1. Introduction

The importance of iron oxides in controlling the fate and transport of many environmentally important radionuclide/metal species has long been recognized in the literature [1–2]. In the storage and disposal of radioactive wastes, which are often envisioned in steel containers, a variety of iron(II) and iron(III) oxides are generated as the container corrodes [3–6]. The presence of iron(II) generates a reducing environment, and in the case of transuranic waste, the actinides are maintained in reduced and therefore less soluble and less mobile forms, for example Pu(III) or (IV) vs. Pu(V) or (VI) [7–13]. To establish a mechanistic understanding of these coupled subsurface redox processes it is necessary to concurrently measure all the pertinent oxidation states of iron and the actinides (metals). These coupled redox reactions are also important for biological redox processes when enzymes, iron and other metals are involved [14]. Microorganisms, by controlling the Fe(II)/Fe(III) ratio, can greatly influence the redox environment ($E_h$) in a way that may define the oxidation state distribution of the multivalent actinides. An understanding of the key mechanisms of redox control in biological systems may also help differentiate between enzymatic and abiotic mechanisms. In this context, a reliable method to quantify the iron oxidation state that is suitable for the complex systems generally encountered under subsurface conditions is of primary importance.

The detection of oxidation states of iron in solutions is most often accomplished using spectrophotometric methods, such as the ferrozine assay [15] and the 1,10-phenanthroline method [16]. Both techniques rely on the formation of a colored $Fe^{2+}$-complex at almost neutral pH and its absorbance is measured in relation to a set of standard solutions. However, these spectrophotometric methods can be limited by the presence of strong complexing agents and possibly by other interferences (e.g., competition with other divalent cations or strongly absorbing co-contaminants). A further obstacle in the $Fe^{2+}$ determination that is often neglected is effect of residual oxygen in the reagents used, especially when the concentration of $Fe^{2+}$ is lower than 0.5 mM.

A common method for the separation of metal ions having different oxidation states is solvent extraction [17–21]. The solvent extraction technique is based on the distribution of the analyte of interest between two immiscible liquids, usually an aqueous solution and an organic solvent containing an extracting species. Many organic molecules were developed and utilized in the extraction process. On the basis of our experience in separation science, bis(2-ethylhexyl) hydrogen phosphate (HDEHP) was chosen as the extractant for this study since it was used to separate various metals including americium, curium, neptunium, plutonium and uranium [22–28]. This acidic extractant allows separation of cations in acidic medium. This extraction approach is likely able to deal with interferences from strong complexants such as NTA (nitrylotriacetate) and citrate, and to a lesser extent EDTA, which can co-exist with radionuclide contaminants in environmental biologically active systems.

In this context, the aim of this paper is to define a solvent extraction procedure to detect ferrous and ferric iron with a view to coupling this method to the detection of different oxidation

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states of multivalent radionuclides, such as uranium, neptunium or plutonium. The extraction method could permit the simultaneous determination of all metal/actinide species using one analytical approach. Additionally, we modified the ferrozine assay [29] for the detection of $Fe^{2+}$ to extend its applicability to these complex systems and use this analytical approach to confirm the results obtained in the HDEHP extraction approach.

## 2. Experimental

The following reagents were used in the present work: ferrozine monosodium salt of 97% purity supplied by Aldrich, bis(2-ethylhexyl)hydrogen phosphate (HDEHP) 97% purity, also from Aldrich, solvents: cyclohexane, toluene and *n*-heptane, all of HPLC grade from Fisher Scientific, HCl (certified ACS Plus) from Fisher Scientific, nitrylotriacetic acid (NTA) 99+ % from Aldrich, sodium dihydrogen citrate 99% from Aldrich, EDTA disodium salt dihydrate 99+ % from Sigma-Aldrich, sodium L-lactate approx. 98% from Sigma-Aldrich, lactate reagent kit from Trinity Biotech., hydroxylamine hydrochloride 99% Reagent Plus from Sigma-Aldrich.

All solution preparation and experiments involving $Fe^{2+}$ were performed in a nitrogen negative pressure anoxic glovebox (MBraun Labmaster 130 with re-circulating copper shaving oxygen purification system) equipped with an oxygen sensor. The oxygen levels in the glovebox were maintained below 3 ppm $O_2$ at all times and were typically < 0.1 ppm $O_2$. It was found that at or below this concentration of oxygen, significant (~1%) oxidation of $Fe^{2+}$ did not occur in the timeframe of a couple of months.

Ferric and ferrous iron solutions were prepared in 0.1 M hydrochloric acid to prevent precipitation of the oxide phases. With the exception of the ferrous iron solution, which was prepared in the glovebox, all solutions were prepared outside the glovebox and transferred into the box, where they were purged to remove oxygen over several weeks by equilibrating with the nitrogen atmosphere. The ferrous solution was prepared with a fresh $FeCl_2$ solid (Sigma, analytical grade) and the ferric solution was prepared using the certified iron(III) oxide (Alfa Aesar stock# 44666, lot# H21R005). Since a standard reference for the iron oxidation state mixture is commercially unavailable, these solutions were used as secondary standards for the preparation of iron oxidation state mixtures with different ferrous to ferric ratios. The oxidation states and iron concentrations in these standards were measured using the ferrozine method, a combination of extraction with ICP-MS assay and direct ICP-MS measurements. The $Fe^{2+}$ concentration in the secondary standard solution was equal to $58.9 \pm 0.3$ mM and the $Fe^{3+}$ concentration in the secondary standard solution was equal to $99.7 \pm 0.4$ mM. These two solutions were kept in a nitrogen-controlled atmosphere and were used for further dilutions.

Aqueous metal concentrations were determined by ICP-MS (Agilent Model 7500ce), fitted with a hydrogen reaction cell, to minimize the interference of the argon–oxygen peak with the iron peak at mass of 56 and to extend the sensitivity of analysis. The ICP-MS determination limit of iron was 5 ppb, which corresponds to an iron concentration of $10^{-7}$ M. The ICP-MS apparatus was calibrated prior to each analytical run. The certified iron standard solution (High Purity Standards) contained 1000 ppm of $Fe^{3+}$ and further dilutions of this sample were used for calibration. Each calibration used a minimum of six points over the iron concentration range of 0–500 ppb. The $R^2$ of calibration linearity was on the level of $0.9999 \pm 0.0001$. Each result reported by ICP-MS was an average of five measurements and the precision of single point measurement was better than 0.5%. The highest relative difference between the synthetic samples having initially the same composition was 9% and was caused by an experimental uncertainty of the extraction and stripping steps.

Considerable experimental work was carried out to obtain the optimum conditions that would enable both ferric and ferrous iron to be determined in aqueous solution and in the presence of complexing ligands such as citrate, NTA and EDTA. The effects of acid concentration for the extraction and stripping steps, contact time for extraction and type of solvent were investigated. Once the procedure was established, a series of experiments were performed to assess its performance in mixed oxidation state solutions and in more complex "real" experimental systems.

### 2.1. Modified ferrozine assay

A modified version of the ferrozine assay [29] was used to crosscheck the results of the extraction experiments. Briefly, 0.9 mL of $4.06 \times 10^{-4}$ M (0.2 g $L^{-1}$) ferrozine in 0.25 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (enzyme grade) was added to 0.1 mL of acidified sample (0.5 M HCl) and the purple color was allowed to develop. The absorbance of the complex was measured *within* 15 minutes at 562 nm, using either a CARY 5000 spectrophotometer or a Thermo Spectronic GeneSys 20 spectrophotometer, and compared to a calibration curve obtained from a series of standards prepared in a similar fashion. No difference in absorption readings was noted between the two instruments. The molar extinction coefficient for the ferrous ferrozine complex was calculated as the average of several calibrations and was equal to $27\,400 \pm 1000$, in good agreement with the literature value [15] of 27 900. A good linear dependence of the Beer–Lambert law was observed for $Fe^{2+}$ concentrations ranging from 10 to 80 μM of $Fe^{2+}$ with the precision of the $Fe^{2+}$ determination being equal to ± 2 μM. The determination limit was assessed to be on the level of 6 μM $Fe^{2+}$.

For samples containing up to 8 mM of iron, the total iron concentration was determined as follows: to 0.1 mL of sample 0.9 mL of 0.28 M hydroxylamine hydrochloride in 0.28 M HCl was added. After 30 minutes, all the $Fe^{3+}$ was reduced and 0.1 mL of this solution was added to 0.9 mL of the ferrozine solution and the absorbance measured *after* 20 minutes. The $Fe^{3+}$ concentration was determined by the difference between the total iron concentration and the $Fe^{2+}$ concentration directly measured in the sample.

### 2.2. Procedure for the HDEHP solvent extraction method

The following procedure is applicable to sample aliquots containing up to 5 mM of ferric iron. The analytical range for $Fe^{2+}$ was assessed and it was determined that up to 8 mM $Fe^{2+}$ could be detected, although it is believed that this amount could be much higher given that $Fe^{2+}$ does not partition into the organic phase. The following procedure is also applicable to solutions containing citrate and NTA at concentrations up to 5 times that of iron. All steps were carried out in glass vials with PTFE lids.

*Step 1*: To 0.5 mL of sample, 1.5 mL of 0.67 M HCl was added to obtain a final acid concentration of 0.5 M. An aliquot of the sample was removed for analysis to determine the total iron concentration.

*Step 2*: To the remaining sample, an equal volume of 0.1 M HDEHP in *n*-heptane was added and shaken for 1 hour. The phases were allowed to completely separate and an aliquot of the aqueous phase was removed for determination of $Fe^{2+}$ content, taking care not to contaminate the extracted portion of the solution with the organic phase.

*Step 3*: To a portion of the organic phase, an equal volume of 4 M HCl was added and shaken for 15 minutes. The phases were allowed to completely separate and an aliquot of the aqueous phase was removed for determination of $Fe^{3+}$ content, again taking care to minimize contamination by the organic phase.

Total iron, ferrous and ferric, concentrations were determined directly from the analysis of the acidified sample by ICP-MS measurements.

### 2.3. Coupled effects of both oxidation states and complexing ligands

A series of solutions containing a total iron concentration of 0.5 mM were prepared using the secondary standard solutions described above and were treated as a reference material since concentrations of $Fe^{2+}$ and $Fe^{3+}$ were precisely known. In the absence of complexing ligands, the $Fe^{2+}$ content was varied from 0 to 0.5 mM. In the presence of citrate, NTA or EDTA, the ratio of $Fe^{2+}$ to $Fe^{3+}$ was kept constant at 1:1 and the concentration of the ligands was varied from 0.05 to 2.5 mM, giving a total iron to ligand ratio of 1:0.1 to 1:5.

### 2.4. Reduction of $Fe^{3+}$ by Shewanella alga under anoxic conditions

Full details of the experimental procedure can be found in Reed *et al.* [14]. Under anaerobic conditions, aqueous $Fe^{3+}$, as a stabilized $Fe^{3+}$-NTA complex was added, at an approximate concentration of 6 mM, to a solution containing the metal-reducing bacteria *S. alga* (strain *BrY*), which had been grown anarobically [30]. Lactate, as sodium L-lactate, was used as the electron donor. Total iron, ferric and ferrous iron concentrations were measured as the experiment progressed using both methods as described above. Lactate was analyzed using a lactate reagent and standards kit (Trinity Biotech) based on the colorimetric technique as recommended by the manufacturer.

## 3. Results and Discussion

The experimental results we report were obtained for solutions that were carefully degassed to remove all trace levels of oxygen. We note that the presence of even trace-levels of oxygen can lead to significantly higher errors when the total Fe concentrations are 0.1 mM or below due to the oxidation of $Fe^{2+}$ by this residual oxygen content leading to spurious and inconsistent analytical results.

### 3.1. Experimental refinement of the HDEHP solvent extraction method

HDEHP is an acidic extractant, thus the distribution ratio of the metal, defined as the ratio of metal in the organic phase to metal in the aqueous phase, depends on the acidity in the aqueous phase. Therefore, the effect of the acid concentration on the extraction of 0.4 mM solutions of ferric and ferrous iron using 0.1 M HDEHP was measured. Between 0.05 M and 1.0 M hydrochloric acid concentration, the extraction of $Fe^{3+}$ into the organic phase was essentially quantitative (>98.5%). Above 1.0 M HCl, the amount of $Fe^{3+}$ extracted decreased rapidly and was almost zero at 4 M. In the case of $Fe^{2+}$, less than $8 \pm 1\%$ was extracted into the organic phase at all acid concentrations investigated. Consequently, it was decided that acidifying the solutions to 0.5 M HCl would provide the optimum conditions for separation of $Fe^{3+}$ from $Fe^{2+}$. Under these conditions, the $Fe^{3+}/Fe^{2+}$ separation factor is $1000 \pm 150$. These results are in agreement with previous studies that have demonstrated that HCl concentrations below 1.0 M give the best separation between $Fe^{3+}$ and either $Fe^{2+}$ [31] or divalent cobalt and nickel, which behave similarly to $Fe^{2+}$ [32].

The extraction of $Fe^{3+}$ into the organic phase (Fig. 1) is relatively slow compared to other metals; for example, the trivalent lanthanides, trivalent actinides, and U(VI) were extracted in minutes [33–35]. Almost quantitative partitioning (greater than 98.5%) of $Fe^{3+}$ occurs after 1 hour and is in agreement with previous studies

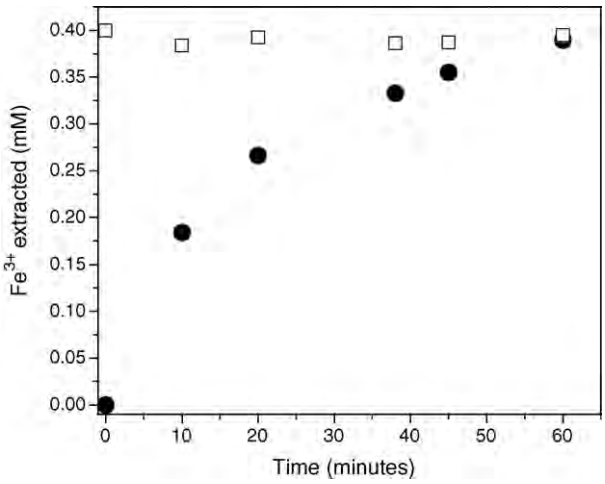


**Fig. 1.** Effect of time on the extraction of 0.4 mM $Fe^{3+}$ from 0.5 M HCl by 0.1 M HDEHP in *n*-heptane. Squares represent total $Fe^{3+}$ (sum in aqueous and organic phases) and circles represent $Fe^{3+}$ extracted into the organic phase.

[36–37]. Extraction rate can be increased by increasing the temperature [33]; however the effect of temperature was not investigated in the present study.

The effects of three solvents, cyclohexane, toluene, and *n*-heptane, on the extraction of 0.4 mM $Fe^{3+}$ from a 0.5 M HCl solution by 0.1 M HDEHP were also established (Table 1). The relative polarities of these solvents are: cyclohexane = 0.006, *n*-heptane = 0.012, and toluene = 0.099 [38]. Altering the solvents has a small but significant effect on the rate of partitioning of $Fe^{3+}$ into the organic phase. When cyclohexane is the solvent, the partitioning occurs on the same timescale as *n*-heptane, i.e., one hour. Toluene appears to increase the time for $Fe^{3+}$ partitioning; after one hour only 75% of the $Fe^{3+}$ has transferred into the organic phase. This trend is correlated with the polarity of the solvents. In solvents of low polarity, HDEHP *via* the formation of dimers [39] extracts trivalent cations according to the following mechanism [40]:

$$M^{3+}{}_{(aq)} + 3(HDEHP)_{2(org)} \leftrightarrow M[H(DEHP)_2]_{3(org)} + 3H^{+}{}_{(aq)} \quad (1)$$

Cyclohexane and *n*-heptane have low and comparable polarities that enable HDEHP to form dimers more rapidly, which leads to a more rapid extraction of $Fe^{3+}$. Toluene, however, with its somewhat higher polarity, causes a decreased rate of $Fe^{3+}$ extraction.

The $Fe^{3+}$ extraction yield is a function of free HDEHP concentration and decreases with increasing $Fe^{3+}$ concentration in the organic phase. Fig. 2 shows the isotherm for $Fe^{3+}$ extraction. The extraction efficiency of $Fe^{3+}$ is significantly influenced by its initial concentration in the aqueous phase. As mentioned before, the extraction is better than 98.5% for $Fe^{3+}$ concentrations below 1 mM. A 5 mM initial concentration leads to the extraction of 92% $Fe^{3+}$. The amount of $Fe^{3+}$ extracted decreases as the initial concentration increases; 77% of $Fe^{3+}$ is extracted at an initial concentration of 10 mM, and 48% of $Fe^{3+}$ is extracted at 35 mM. From the data

**Table 1**
Effect of different solvents on the $Fe^{3+}$ extraction by 0.1 M HDEHP measured for various extraction times. Errors represent one standard deviation from the mean value.

| Time (minutes) | % $Fe^{3+}$ extracted into organic phase | | |
|---|---|---|---|
| | Cyclohexane | *n*-Heptane | Toluene |
| 10 | 35.92 ± 0.45 | 47.94 ± 0.88 | 32.38 ± 0.64 |
| 20 | 56.78 ± 2.67 | 67.86 ± 2.12 | 46.84 ± 1.22 |
| 30 | 71.84 ± 1.97 | 86.11 ± 0.20 | 51.78 ± 0.45 |
| 45 | 84.53 ± 0.03 | 91.70 ± 0.19 | 62.46 ± 0.14 |
| 60 | 93.68 ± 0.03 | 98.68 ± 0.30 | 75.04 ± 0.26 |

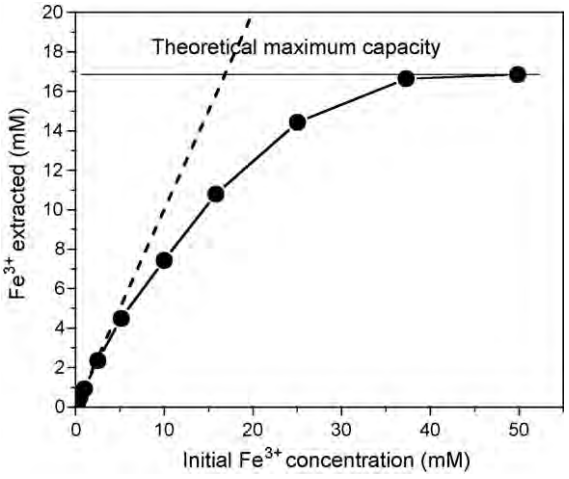


**Fig. 2.** Extraction capacity of 0.1 M HDEHP in *n*-heptane. The amount of extracted $Fe^{3+}$ (circles) was measured after the back-extraction from the organic phase. The dotted line represents the ideal case (100% of $Fe^{3+}$ extraction) where the capacity of the HDEHP is not limiting. The theoretical maximum capacity of 0.1 M HDEHP for $Fe^{3+}$ is shown by the solid line.

presented in Fig. 2, the organic phase is saturated at an initial $Fe^{3+}$ concentration of 35 mM, with only ~17 mM extracted. In these experiments, an HDEHP concentration of 100 mM was used. Since six HDEHP molecules are required to extract one $Fe^{3+}$ cation (see Eq. (1)), this concentration of HDEHP allows a maximum of 16.7 mM $Fe^{3+}$ to be extracted. The net effect of this $Fe^{3+}$ "capacity" is that there will be a limiting $Fe^{3+}$ concentration that depends on the concentration of HDEHP used in the organic phase, and this limit will, in part, define the error in the analytical approach. Therefore, under the conditions of the procedure presented here, a maximum concentration of 5 mM $Fe^{3+}$ is recommended, which results in an error of less than 10%. To measure higher concentrations of $Fe^{3+}$, the sample could be diluted or a higher concentration of HDEHP could be used up to a maximum of 0.3 M.

For the range of concentrations investigated, the $Fe^{2+}$ remains in the aqueous phase and does not interfere with the extraction of $Fe^{3+}$, which is discussed in Section 3.2. For this reason, the $Fe^{2+}$ concentration limit in this extraction procedure is defined simply by its solubility in the aqueous phase.

### 3.2. Mixed oxidation state system and the effect of highly complexing ligands

In all the experiments reported herein, the ferrous and ferric secondary standard solutions were used as the reference. The separation of iron oxidation states in the synthetic mixtures and analytical sample preparations were conducted in accordance with the modified ferrozine method and the extraction method using our anoxic nitrogen glovebox. The results of the experiments performed on mixed $Fe^{2+}/Fe^{3+}$ oxidation state systems using these two methods are shown in Fig. 3. The total iron concentrations measured by ICP-MS were always greater than those determined by the ferrozine method. The explanation for this observation is that the reduction of $Fe^{3+}$ was not complete. However, agreement to within our target experimental error could be achieved. There is also a slight difference between the two methods in the determination of $Fe^{3+}$ concentration, since the solvent extraction method determines the concentration directly, whereas the ferrozine assay determines the concentration by difference. The results obtained by both methods, however, agree to within $7 \pm 4\%$.

In the presence of citrate and NTA (Table 2), the detection of both oxidation states is not significantly affected. However, the presence

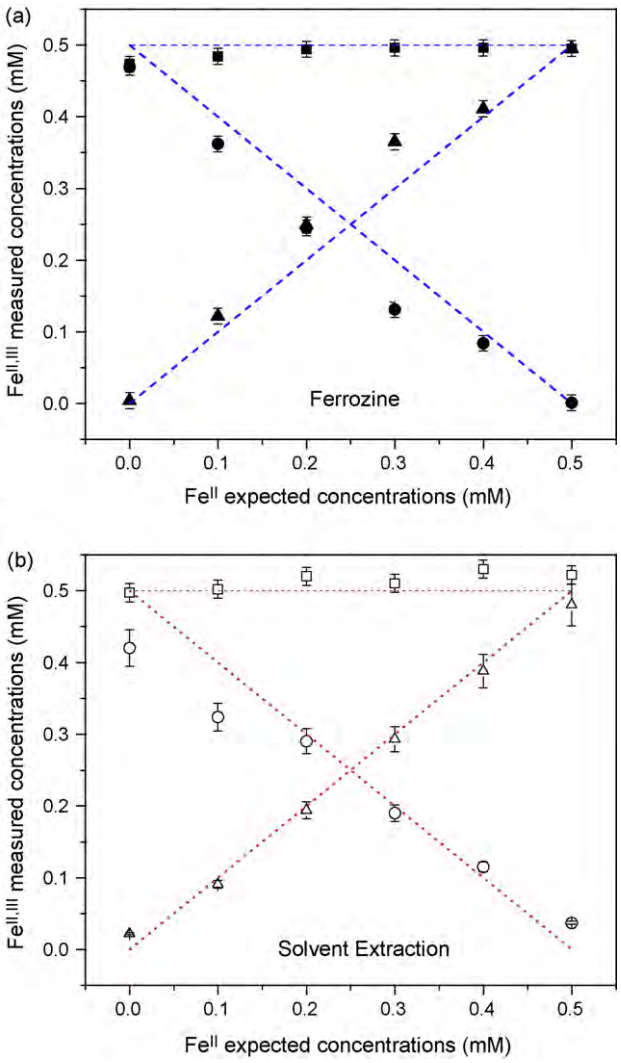


**Fig. 3.** Comparison of the results obtained from the ferrozine assay (a) and solvent extraction method (b) at $Fe_{total}$ concentration = 0.5 mM. Fe total data are represented by squares, $Fe^{2+}$ by triangles, $Fe^{3+}$ by circles and dashed lines represent the true values.

**Table 2**
Effect of citrate, NTA and EDTA on the detection of iron by the solvent extraction method (SE) and the ferrozine assay (FA). Total iron concentration = 0.5 mM, with a 1:1 ratio of $Fe^{2+}$ to $Fe^{3+}$. The ratio of total iron to ligand varied from 1:0.1 to 1:5.

| System | Fe:ligand ratio | Total iron (mM) | | $[Fe^{2+}]$ (mM) | | $[Fe^{3+}]$ (mM) | |
|---|---|---|---|---|---|---|---|
| | | SE | FA | SE | FA | SE | FA |
| Fe/citrate | 1:0.1 | 0.518 | 0.576 | 0.209 | 0.265 | 0.232 | 0.311 |
| | 1:1 | 0.518 | 0.597 | 0.222 | 0.248 | 0.263 | 0.349 |
| | 1:2 | 0.528 | 0.600 | 0.242 | 0.269 | 0.278 | 0.331 |
| | 1:5 | 0.567 | 0.567 | 0.259 | 0.321 | 0.290 | 0.246 |
| Fe/NTA | 1:0.1 | 0.529 | 0.498 | 0.250 | 0.270 | 0.276 | 0.228 |
| | 1:1 | 0.512 | 0.499 | 0.242 | 0.247 | 0.244 | 0.252 |
| | 1:2 | 0.516 | 0.484 | 0.228 | 0.254 | 0.235 | 0.230 |
| | 1:5 | 0.524 | 0.508 | 0.274 | 0.258 | 0.256 | 0.249 |
| Fe/EDTA | 1:0.1 | 0.517 | 0.455 | 0.244 | 0.270 | 0.252 | 0.185 |
| | 1:1 | 0.519 | 0.258 | 0.320 | 0.242 | 0.158 | 0.015 |
| | 1:2 | 0.515 | 0.235 | 0.368 | 0.230 | 0.141 | 0.005 |
| | 1:5 | 0.500 | 0.224 | 0.411 | 0.217 | 0.109 | 0.007 |

**Table 3**
Log *K* values for the complexes formed between citrate, NTA or EDTA with $Fe^{2+}$ or $Fe^{3+}$. Values are for $I = 0.1$ M at 25 °C for [ML]/[M][L] [41].

| Ligand | log *K* | |
|---|---|---|
| | $Fe^{2+}$ | $Fe^{3+}$ |
| Citrate | 4.62 | 11.2 |
| NTA | 8.90 | 16.0 |
| EDTA | 14.3 | 25.1 |

of EDTA affects both of these methods with the influence increasing with increasing EDTA concentration. In the HDEHP solvent extraction procedure, the determination of total iron concentration in the presence of EDTA is unaffected but the measurement of the relative concentration of each oxidation state is affected. More specifically, the measured concentration of $Fe^{2+}$ is somewhat elevated whereas the concentration of $Fe^{3+}$ is lower than the value expected. In the extractions carried out on systems containing EDTA and only one iron oxidation state, the presence of EDTA did not affect $Fe^{2+}$ determination. However, EDTA caused $Fe^{3+}$ to remain in the aqueous phase during the initial extraction step. In the solvent extraction method, EDTA interferes with the formation of $Fe^{3+}$–HDEHP dimers, thus prevents the complete extraction of $Fe^{3+}$ into the organic phase, resulting in a lower $Fe^{3+}$ concentration than expected. Altering the experimental conditions, for example increasing the acid concentration and increasing the time for partitioning to occur in the initial extraction step, did not resolve this problem. Therefore, in the mixed oxidation state system, the apparent increase in $Fe^{2+}$ concentration is due to $Fe^{3+}$ that remained in the aqueous phase, which led to a correspondingly lower $Fe^{3+}$ concentration in the organic phase.

In the ferrozine assay, the measurement of $Fe^{2+}$ was unaffected by the presence of EDTA but the total iron measurement and therefore the determination of $Fe^{3+}$ content were impacted. Experiments performed on systems containing EDTA and only one oxidation state of iron (results not shown) revealed that hydroxylamine hydrochloride was unable to quantitatively reduce $Fe^{3+}$ to $Fe^{2+}$ because of EDTA complexation and as a result the $Fe^{3+}$ concentration determined was lower than expected. The effect of EDTA on both these methods can be explained in terms of differences in stability constants. The strength of the complexes formed between $Fe^{2+}$ or $Fe^{3+}$ and the three ligands investigated in this study are compared in Table 3. The $Fe^{3+}$-EDTA complex is much stronger than the other $Fe^{3+}$ organic complexes as well as all the $Fe^{2+}$ organic complexes.

In summary, for the range of experimental conditions investigated, both the solvent extraction procedure and the ferrozine method could not overcome the effects of strong EDTA complexation to permit reliable detection of $Fe^{3+}$. Determination of ferrous and ferric iron in the presence of EDTA could, however, be done by using ICP-MS to measure the total iron concentration and the ferrozine method to establish the $Fe^{2+}$ content.

### 3.3. Reduction of $Fe^{3+}$ by *S. alga* under anoxic conditions

The analytical procedures described herein to determine different oxidation states of iron were applied to biologically active environmental samples in the presence of a moderately strong complexant, NTA. Fig. 4 shows the reduction of $Fe^{3+}$ (as an NTA complex) to $Fe^{2+}$ by *S. alga* that was performed in separate experiments. It was impractical, due to the time-intensive nature of the analyses in the glovebox, to perform the ferrozine method and extraction simultaneously. There is an excellent correlation between the utilization of lactate, as an electron donor, and the reduction of $Fe^{3+}$. There is a strong relationship between the production of $Fe^{2+}$ and the growth of the cells over the course of the experiment (data not shown). Both the solvent extraction procedure and the ferrozine method, Fig. 4a and 4b respectively, were able to track the reduction of $Fe^{3+}$ to $Fe^{2+}$ over the course of the experiments and gave very similar results. This agreement in a relatively complex media application confirms the applicability of both these analytical methods to the oxidation state specific analysis of iron in environmental media.

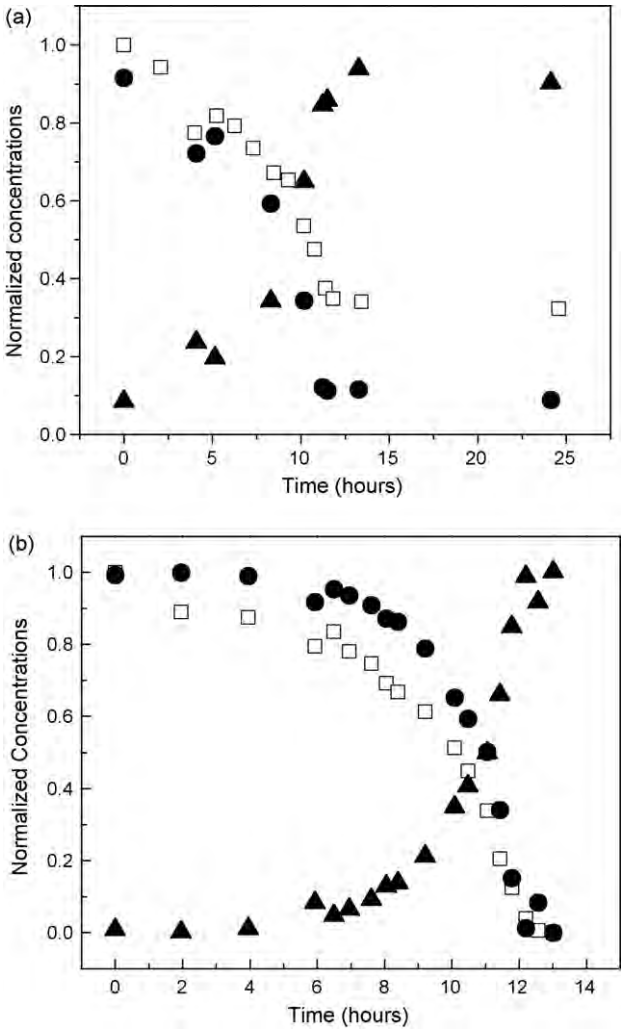


**Fig. 4.** Bioreduction of $Fe^{3+}$ (circles), initially present as $Fe^{3+}$-NTA, to soluble $Fe^{2+}$ (triangles) by *S. alga*. Lactate was utilized as the electron donor. Iron concentrations were determined using (a) the HDEHP solvent extraction method and (b) the ferrozine method. Lactate concentration (squares) was determined using a lactate reagent kit.

## 4. Conclusions

The simultaneous measurement of $Fe^{2+}$ and $Fe^{3+}$ is needed to establish and understand key redox processes in complex environmental systems and must be done very carefully to get the correct results. All reagents used in the analytical procedure must be oxygen free, since residual oxygen will easily oxidize trace-levels of $Fe^{2+}$ and introduce significant error into the analysis. When the $Fe^{2+}$ concentration is lower than 0.1 mM, this error can reach 100% in the ferrozine and extraction methods indicating that the ferrous ions have disappeared from the system. Alternatively, the presence of a strong complexing agent in the system can mask the $Fe^{3+}$ concentration leading to an overestimation of the $Fe^{2+}$ content. For these reasons, an incorrect result in the $Fe^{2+}$ and $Fe^{3+}$ determination is obtained if analytical conditions are not carefully controlled.

The results presented herein show that a solvent extraction procedure that utilizes HDEHP for the separation of ferrous and ferric iron in aqueous solutions, when combined with ICP-MS analysis, can provide at least the same degree of accuracy and sensitivity as the ferrozine method. This approach has the added benefit of also separating oxidation states of multivalent actinides so that it can be combined with radioanalytical methods (e.g., liquid scintillation counting) to analyze these oxidation states at the same time. Both the ferrozine and HDEHP extraction methods were capable of detecting both oxidation states of iron in systems containing complexing agents with log $K$ values less than 17, in this case citrate and NTA, and in a more complex system containing microbes.

However, the presence of EDTA affected the oxidation-specific analysis of iron in both methods. The extent of this effect increased with increasing EDTA concentration. In the solvent extraction procedure, total iron concentration was unaffected, whereas the $Fe^{2+}$ concentration was higher than expected and the $Fe^{3+}$ lower than expected. In the ferrozine method, $Fe^{2+}$ concentration was unaffected but the total iron and thus $Fe^{3+}$ concentrations were lower than expected. In both cases, this is attributed to the strength of the $Fe^{3+}$-EDTA complex (log $K$ = 25.1). On the basis of these observations, we proposed to use the following method in the presence of EDTA: determine the total iron concentration directly from the sample using ICP-MS, equipped with a hydrogen reaction cell, determine the $Fe^{2+}$ concentration, using the modified ferrozine method we described, and determine the $Fe^{3+}$ concentrations by difference. This procedure has broad applicability and is independent of the presence of strong chelating ligands in the aqueous solution.

Future work is focused on extending the applicability of these procedures to complex environmental systems and developing a co-extraction approach when multivalent actinides are also present.

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