APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1987, p. 1536–1540
0099-2240/87/071536-05$02.00/0

Vol. 53, No. 7

Rapid Assay for Microbially Reducible Ferric Iron in Aquatic Sediments

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Received 9 February 1987/Accepted 21 April 1987

The availability of ferric iron for microbial reduction as directly determined by the activity of iron-reducing organisms was compared with its availability as determined by a newly developed chemical assay for microbially reducible iron. The chemical assay was based on the reduction of poorly crystalline ferric iron by hydroxylamine under acidic conditions. There was a strong correlation between the extent to which hydroxylamine could reduce various synthetic ferric iron forms and the susceptibility of the iron to microbial reduction in an enrichment culture of iron-reducing organisms. When sediments that contained hydroxylamine-reducible ferric iron were incubated under anaerobic conditions, ferrous iron accumulated as the concentration of hydroxylamine-reducible ferric iron declined over time. Ferrous iron production stopped as soon as the hydroxylamine-reducible ferric iron was depleted. In anaerobic incubations of reduced sediments that did not contain hydroxylamine-reducible ferric iron, there was no microbial iron reduction, even though the sediments contained high concentrations of oxalate-extractable ferric iron. A correspondence between the presence of hydroxylamine-reducible ferric iron and the extent of ferric iron reduction in anaerobic incubations was observed in sediments from an aquifer and in fresh- and brackish-water sediments from the Potomac River estuary. The assay is a significant improvement over previously described procedures for the determination of hydroxylamine-reducible ferric iron because it provides a correction for the high concentrations of solid ferrous iron which may also be extracted from sediments with acid. This is a rapid, simple technique to determine whether ferric iron is available for microbial reduction.

Defining the zones in which microbial reduction of ferric iron [Fe(III)] influences the iron geochemistry of surface water and groundwater systems can be hampered by the difficulty of making direct measurements of the rates of Fe(III) reduction. An Fe(III) reduction rate can be estimated by measuring the accumulation of ferrous iron [Fe(II)] over time in anaerobic incubations (5, 11). However, in situ conditions are difficult to replicate in such incubations, and thus the Fe(III) reduction rate that is measured must be viewed as a potential rate rather than the in situ rate. The incubation technique is not readily applicable to oligotrophic environments such as some aquifer systems in which the rates of organic matter decomposition are so slow that an incubation period of a year or more might be required before increases in Fe(II) could be detected. The incubation technique is also labor-intensive.

Given the limitations of the incubation technique, a simple chemical assay that could identify zones in which microbially reducible Fe(III) is available might be preferable as an initial screening test. If microbially reducible Fe(III) is present, then there is the potential for Fe(III) reduction, and further investigation on microbial Fe(III) reduction may be warranted.

Fe(III) exists in a variety of chemical forms, spanning a continuum from highly crystalline minerals to material with little or no crystalline structure (10). Bacteria preferentially reduce amorphous Fe(III) oxyhydroxides (5, 7, 8). The most commonly used method to measure amorphous Fe(III) oxyhydroxides in soils and sediments is the acid ammonium oxalate extraction technique (9). Although acid ammonium oxalate extracted the microbially reducible Fe(III) in freshwater sediments of the Potomac River, high concentrations of Fe(III) that were not available for microbial reduction were also extracted (5). Less than 20% of the oxalate-extractable FE(III) in the surface sediments was available for microbial reduction, and in deeper sediments, none of the nearly 300 μmol of oxalate-extractable Fe(III) per g of dry sediment was reduced during anaerobic incubations (5).

In an evaluation of techniques for the extraction of amorphous Fe(III) oxyhydroxide from soils, Chao and Zhou (3) concluded that the reduction of Fe(III) with 0.25 M hydroxylamine hydrochloride in 0.25 M HCl at 50°C for 30 min was the extraction method of choice. The hydroxylamine extraction was more selective for amorphous Fe(III) oxyhydroxide than the acid ammonium oxalate extraction was because hydroxylamine extracted much less magnetite. This is an important consideration because it has been hypothesized that the oxalate-extractable Fe(III) that is resistant to microbial reduction in sediments is in the form of mixed Fe(III)-Fe(II) compounds (5). Thus, hydroxylamine-reducible Fe(III) might more closely correspond with microbially reducible Fe(III) than with oxalate-extractable Fe(III). However, the method of Chao and Zhou does not distinguish between hydroxylamine-reducible Fe(III) and Fe(II) that is extracted under acidic conditions. Anaerobic sediments can contain substantial quantities of solid Fe(II) forms that are soluble in acid (5). Thus, the method of Chao and Zhou significantly overestimates the concentration of amorphous Fe(III) oxyhydroxide in such reduced environments.

This paper describes a hydroxylamine extraction method that is selective for microbially reducible Fe(III). The method is fast, simple, and sensitive and has been found to be applicable to the study of fresh- and brackish-water surface sediments as well as aquifer sediments.

MATERIALS AND METHODS

The hydroxylamine extraction method of Chao and Zhou (3) was modified so that the Fe(III) that was reduced and

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extracted with the mixture of hydroxylamine hydrochloride in HCl could be distinguished from the Fe(II) that was also extracted from the sediments with this mixture. The extraction temperature and time were also changed.

HCl-extractable Fe(II) in the sediments was measured as previously described (4). Approximately 0.1 g of wet sediment was transferred to 5 ml of 0.5 M HCl in a glass scintillation vial of known weight. The sediment and acid were mixed with gentle swirling for ca. 30 s. The weight of the added sediment was determined. After 1 h at room temperature, a 0.1-ml sample of the extract was added to 5 ml of ferrozine (1 g/liter) in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer at pH 7. After being mixed for 15 s, the mixture was passed through a polycarbonate filter (Nuclepore Corp.; pore diameter, 0.2 μm). The amount of Fe(II) was determined by measuring the A_{562} of the filtrate (12). Fe(II) was not oxidized and Fe(III) was not reduced during the extraction (4).

Another sample of the sediment was extracted by the same procedure as that described above with the exception that the extractant was 5 ml of 0.25 M hydroxylamine hydrochloride in 0.25 M HCl. Under acidic conditions, hydroxylamine reduces Fe(III) to Fe(II) (2).

The amount of hydroxylamine-reducible Fe(III) was calculated as the difference between the Fe(II) measured in the hydroxylamine and HCl extractions.

Measurement of Fe(III) reduction. Sediments (10 to 20 ml) were transferred under N_2-CO_2 (93:7, vol/vol) to 25-ml serum bottles which were then sealed with a butyl rubber stopper (Bellco Glass, Inc.). The gas mixture was passed through a heated column of reduced copper filings to remove any traces of O_2. The incubations were done at 20°C in the dark without shaking. The subsamples were removed under N_2-CO_2 over time with a 1-ml plastic syringe, and HCl-extractable Fe(II) and hydroxylamine-reducible Fe(III) were determined as described above.

Synthetic Fe(III) forms. Fe(III) forms were synthesized or purchased as previously described (5). The amount of hydroxylamine-extractable Fe(III) in the Fe(III) forms was determined as described above. The total amount of iron in the Fe(III) forms was determined by dissolving the Fe(III) forms in concentrated HCl, diluting the solutions with deionized water, and assaying for iron by atomic absorption. For Fe(III)-Fe(II) compounds, the appropriate corrections were made for Fe(II) content in determining the quantity of Fe(III) present.

The ability of an Fe(III)-reducing enrichment culture to reduce the synthetic Fe(III) forms with acetate as the electron donor was determined as described previously (5).

To determine the recovery of synthetic amorphous Fe(III) oxyhydroxide from sediments, a slurry of synthetic amorphous Fe(III) oxyhydroxide was added to sediments.The volume of the slurry was 5 to 10% of the sediment volume. For additions to reduced sediments, the slurry was bubbled with N_2 to remove dissolved O_2, and the slurry was added to the sediments under a stream of N_2-CO_2 (93:7, vol/vol).

Sediment sampling. Sediments were collected from the previously described fresh- and brackish-water sites in the Potomac River estuary (4). For initial development of the method, surface sediments were collected from the freshwater site with an Eckman dredge and transported in canning jars to the laboratory. The sediments were incubated under N_2-CO_2 (93:7, vol/vol) in 1-liter bottles in the dark at 20°C for more than a month to deplete the microbially reducible Fe(III). These sediments were referred to as reduced freshwater sediments. Some of the reduced sediment was oxidized by mixing it with a magnetic stir bar in the air at room temperature for a day or more. The sediments that were exposed to air were referred to as oxidized freshwater sediments.

TABLE 1. Hydroxylamine-reducible Fe(III) and availability of Fe(III) for microbial reduction with various Fe(III) forms

Fe(III) form	% Fe(III)	
	Hydroxylamine reducible[a]	Microbially reducible[b]
Amorphous Fe(III) oxyhydroxide	103	45
Amorphous Fe(III) oxyhydroxide adsorbed to clay	95	37
Goethite (α-FeOOH)	3	5
Fe(III)-Fe(II)[c]	2	1
Fe_2O_3	<1	0
Fe_3O_4	<1	2
$FePO_4 \cdot 2H_2O$	<1	8
Akaganeite (β-FeOOH)	<1	12

[a] Percentage of total Fe(III); mean of duplicate determinations.
[b] Percentage of added Fe(III) reduced to Fe(II) (soluble in 0.5 N HCl) after 55 or more days of incubation with an Fe(III)-reducing acetate enrichment culture; mean of duplicate determinations (data taken from reference 5).
[c] Mixture of Fe(III)-Fe(II) compounds produced from the reduction of amorphous Fe(III) oxyhydroxide in an acetate enrichment culture.

Cores of sediments from the two sites were collected with a gravity coring device as previously described (5). The cores were extruded and sectioned under N_2 (5).

Sediments were collected from a sand and gravel aquifer in Pensacola, Fla. The geochemistry of this aquifer has been described in detail elsewhere (6). Fe(III) reduction is considered a potentially important process in the decomposition of creosote wastes that contaminate portions of the aquifer (1). A sandy sample was collected from a depth of approximately 6 m at a creosote-contaminated site located approximately 55 m southwest of the creosote works recirculation impoundment. Sediment samples were subcored with a cork borer from material on the drill auger flights and transferred to serum bottles under a stream of argon. Within an uncontaminated zone of the aquifer, approximately 470 m east southeast of the impoundment, a core sample was taken from a clay lens at a 6-m depth. The core was placed in a N_2-purged glove bag and subcored with a 3-ml plastic syringe with the end cut off. Subcores were extruded into serum bottles.

RESULTS AND DISCUSSION

Correspondence of hydroxylamine- and microbially reducible Fe(III) in an Fe(III)-reducing culture. There was a strong correlation (r^2 = 0.94) between the extent of reduction of various synthetic Fe(III) forms with hydroxylamine and the capacity of an Fe(III)-reducing acetate enrichment culture to reduce the Fe(III) forms (Table 1). Susceptibility to hydroxylamine reduction was a more selective indicator of microbially reducible Fe(III) than solubility in acid ammonium oxalate. Whereas oxalate extracted Fe(III) forms such as Fe_3O_4 and $FePO_4 \cdot 2H_2O$ which the culture did not extensively reduce (5), hydroxylamine did not reduce these Fe(III) forms.

Without any other information, the results in Table 1 suggest that more of the Fe(III) in amorphous Fe(III) oxyhydroxide was hydroxylamine reducible than was microbially reducible. However, during the microbial reduction of amorphous Fe(III) oxyhydroxide, much of the Fe(III)

TABLE 2. Extraction of sediment Fe(III) and added synthetic amorphous Fe(III) oxyhydroxide in oxidized and reduced freshwater sediments

Sediment type	Amt (μmol/g of dry sediment) of:				% Synthetic Fe(III) recovered[a]
	Synthetic Fe(III)[b]	Fe(II)[c]	Total Fe[d] (mean ± SD)	Fe(III)[e]	
Oxidized	0	0	172 ± 2	172	
	347	0	486 ± 4	486	90
Reduced	0	256 ± 9	256 ± 6	0	
	125	283 ± 10	380 ± 17	97	78

[a] Calculated as the difference in Fe(III) in sediment with and without added synthetic Fe(III) divided by the amount of added synthetic amorphous Fe(III) oxyhydroxide.
[b] Added as amorphous Fe(III) oxyhydroxide.
[c] Measured after extraction with 0.5 M HCl.
[d] Measured after extraction with 0.25 M hydroxylamine HCl in 0.25 M HCl; $n = 5$.
[e] Calculated as total Fe − Fe(II).

was converted to the crystalline, mixed Fe(III)-Fe(II) mineral magnetite (Fe_3O_4) (5). Magnetite was neither hydroxylamine nor microbially reducible (Table 1). Thus magnetite formation consumed much of the amorphous Fe(III) oxyhydroxide that otherwise could have been microbially reduced.

Recovery of Fe(III) and importance of correction for Fe(II). The high concentrations of HCl-soluble Fe(II) in reduced sediments (Table 2) emphasized the importance of distinguishing between hydroxylamine-reducible Fe(III) and HCl-soluble Fe(II). In the reduced sediments, the amount of iron extracted with hydroxylamine in HCl was the same as the amount of Fe(II) extracted by HCl alone (Table 2), indicating that there was no hydroxylamine-reducible Fe(III) in the reduced sediments. With the standard hydroxylamine extraction procedure (3), there would be no indication of whether the iron that was extracted with hydroxylamine was Fe(III) or Fe(II).

Added synthetic amorphous Fe(III) oxyhydroxide was almost completely recovered from oxidized sediments by the extraction procedure (Table 2). The apparent recovery in reduced sediments was lower. However, the concentration of Fe(II) in reduced sediments after the addition of Fe(III) was higher than in reduced sediments without added Fe(III). This difference indicated that some of the amorphous Fe(III) oxyhydroxide that was added to the reduced sediment was reduced to Fe(II) within the time (ca. 0.5 h) between the addition of Fe(III) to the sediment and the start of the extractions. When the increase in Fe(II) over that in sediments without added Fe(III) (27 μmol/g of dry sediment) was subtracted from the quantity of Fe(III) added, the calculated recovery of amorphous Fe(III) oxyhydroxide from reduced sediment was 99%.

Correspondence of hydroxylamine- and microbially reducible Fe(III) in sediments. When a mixture of oxidized and reduced sediments was incubated under anaerobic conditions, there was an accumulation of Fe(II) over time that corresponded with a decline in hydroxylamine-reducible Fe(III) (Fig. 1). The accumulation of Fe(II) stopped as the concentration of hydroxylamine-reducible Fe(III) went to zero. There was no Fe(II) accumulation or loss of hydroxylamine-reducible Fe(III) in mixtures of oxidized and reduced sediments that were autoclaved (121°C, 15 min) prior to incubation (data not shown). These results suggested that hydroxylamine-reducible Fe(III) was microbially reduced to Fe(II) over time. Similar results were obtained with oxidized brackish-water sediments (data not shown).

There was no microbial reduction of Fe(III) in the reduced freshwater sediment, as indicated by a lack of Fe(II) accumulation over time (Fig. 1). Although reduced sediments contained ca. 300 μmol of oxalate-extractable Fe(III) per g of dry sediment (5), they contained no hydroxylamine-reducible Fe(III) (Fig. 1). These results demonstrated that, unlike the oxalate extraction procedure (5), the hydroxylamine extraction procedure did not measure Fe(III) forms that were not available for microbial reduction.

In sediments which contained hydroxylamine-reducible Fe(III), the accumulation of Fe(II) during the reduction of Fe(III) was 60 to 80% greater than the decrease in hydroxylamine-reducible Fe(III) (Fig. 1 and data not shown). This result suggested that hydroxylamine did not reduce all of the microbially reducible Fe(III). The microbially reducible Fe(III) in these sediments was probably amorphous Fe(III) oxyhydroxide (5). The good recovery of synthetic amorphous Fe(III) oxyhydroxide added to sediments suggested that hydroxylamine should reduce most of the natural amorphous Fe(III) oxyhydroxide in the sediment. However, Chao and Zhou (3) reported that whereas hydroxylamine reduced ca. 80% of their synthetic amorphous Fe(III) oxyhydroxide, only ca. 40% of a natural amorphous Fe(III) oxyhydroxide was reduced. A similar inability of hydroxylamine to reduce all of the amorphous Fe(III) oxyhydroxide in sediments may explain the disparity between hydroxylamine- and microbially reducible Fe(III) in the sediments. Although hydroxylamine-reducible Fe(III) was not a completely quantitative estimate of microbially reducible Fe(III), this did not invalidate the method for its

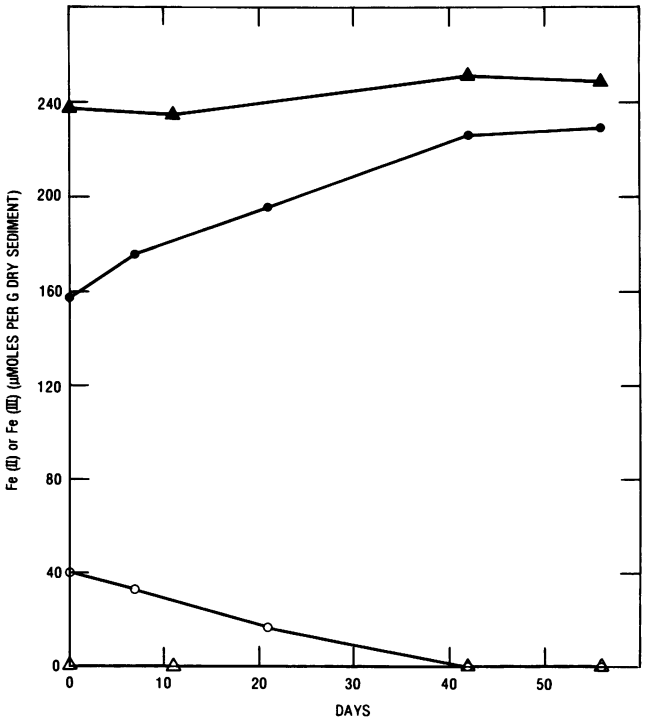

FIG. 1. Hydroxylamine-reducible Fe(III) and HCl-extractable Fe(II) versus time in reduced freshwater sediments and a mixture of approximately equal volumes of oxidized and reduced freshwater sediments incubated under N_2-CO_2. Values shown are the means of duplicate determinations. Symbols: ○ and ●, hydroxylamine-reducible Fe(III) and HCl-extractable Fe(II) in a mixture of oxidized plus reduced sediments; △ and ▲, hydroxylamine-reducible Fe(III) and HCl-extractable Fe(II) in reduced sediment.

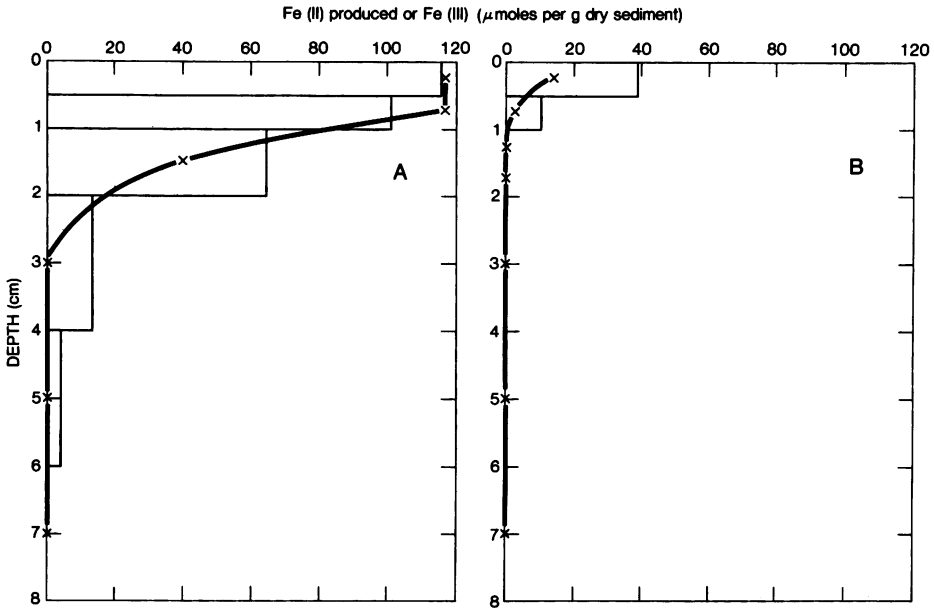

FIG. 2. Distribution of hydroxylamine-reducible Fe(III) (×) and the accumulation of HCl-extractable Fe(II) (bars) in freshwater (A) and brackish-water (B) sediments from the Potomac River estuary. Incubation periods for Fe(II) production were 14 and 16 days for tidal-river and lower-estuary samples, respectively.

primary purpose, which was to identify zones in which Fe(III) was available for microbial reduction. The exhaustive studies of Chao and Zhou (3) demonstrate that development of a technique which extracts all the amorphous Fe(III) oxyhydroxide yet is still selective for amorphous Fe(III) oxyhydroxide may not be possible.

Depth profiles in estuarine sediments. At both the fresh- and brackish-water sites, the depth profiles of the initial concentration of hydroxylamine-reducible Fe(III) in the sediments corresponded with the profiles of the extent of Fe(III) reduction in sediments incubated under anaerobic conditions (Fig. 2). Hydroxylamine-reducible Fe(III) and Fe(III) reduction were highest in the surface sediments and declined with depth. The brackish-water sediments had less microbially reducible Fe(III) than the freshwater sediments. In accordance with previous results (11), inhibition of sulfate reduction in brackish-water sediments with added molybdate (20 mM, final concentration) did not alter the rate of Fe(III) reduction (data not shown).

The decline of hydroxylamine-extractable Fe(III) to nondetectable levels within the upper 2 cm of the sediments at both sites contrasted with the persistence with depth of 125 to 150 μmol (brackish-water site) or 250 to 300 μmol (freshwater site) of Fe(III) per g of dry sediment that was oxalate extractable but resistant to microbial reduction (5; E. J. P. Phillips and D. R. Lovley, Soil Sci. Soc. Am. J., in press). These results further emphasized that the hydroxylamine reduction procedure was more selective for microbially reducible Fe(III) than the oxalate extraction method.

Aquifer sediments. The hydroxylamine extraction method was also applicable to sediments from the creosote-contaminated aquifer in Pensacola, Fla. (Fig. 3). There was a low concentration of hydroxylamine-reducible Fe(III) in the sediments. During anaerobic incubations, hydroxylamine-reducible Fe(III) declined over time, with the concurrent accumulation of Fe(II). As hydroxylamine-reducible Fe(III) was depleted, Fe(III) reduction stopped. These results indicated that there was Fe(III) in the sediments at this site which could serve as an electron acceptor for microbial metabolism under anaerobic conditions. This finding is in accordance with the hypothesis (1) that Fe(III) reduction is an important process for the decomposition of creosote wastes in this portion of the aquifer.

Sediments from a clay lens within an uncontaminated site of the Pensacola aquifer had higher initial concentrations of HCl-extractable Fe(II) (45 μmol/g [dry weight]), but no hydroxylamine-reducible Fe(III). There was also no microbial Fe(III) reduction, since no Fe(II) accumulated over 50 days of anaerobic incubation (data not shown).

In summary, the results demonstrate that this newly developed hydroxylamine extraction procedure is a useful indicator of the availability of microbially reducible Fe(III)

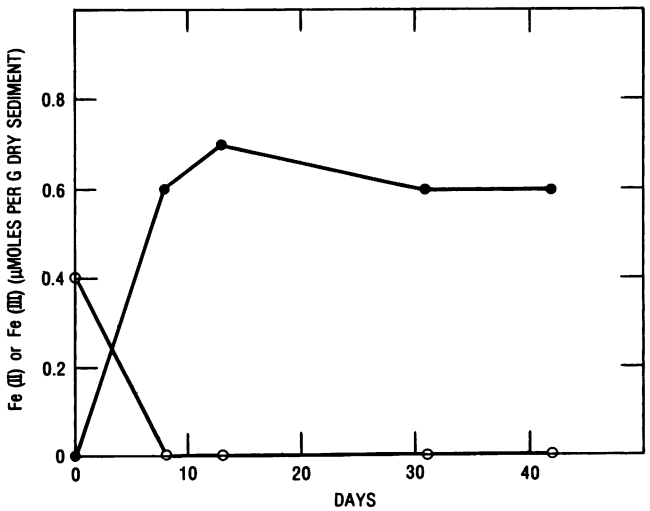

FIG. 3. Hydroxylamine-reducible Fe(III) (○) and HCl-extractable Fe(II) (●) versus time in sediments from a creosote-contaminated aquifer that were incubated under anaerobic conditions.

in sediments. The procedure is rapid, simple, and highly selective for microbially reducible Fe(III). In contrast to the laborious technique of anaerobically incubating sediments and measuring the accumulation of Fe(II), the hydroxylamine extraction procedure indicates within hours whether a sample contains microbially reducible Fe(III). The method should also aid in the study of other aspects of Fe(III) geochemistry when an estimate of the concentration of amorphous Fe(III) oxyhydroxide is desired because, in contrast to the standard hydroxylamine extraction method, the procedure described here differentiates between Fe(III) reduced by hydroxylamine and the Fe(II) that is also extracted under acidic conditions.

ACKNOWLEDGMENTS

We thank Mary Jo Baedecker and Mike Godsy for their help in obtaining samples from the aquifer, William Andrle and Edward Callender for help in collecting the sediment from the Potomac River, and Richard Smith for his helpful suggestions on the manuscript.

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