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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE REDUCTION OF URANIUM BY SULFATE-REDUCING BACTERIA IN THE TERRESTRIAL SUBSURFACE AND A PURE CULTURE BACTERIUM

A DISSERTATION

Submitted to the graduate faculty in partial fulfillment of the requirements of the degree

of

DOCTOR OF PHILOSOPHY

By

Dwayne Anthony Alexander Elias

Norman, OK

2002

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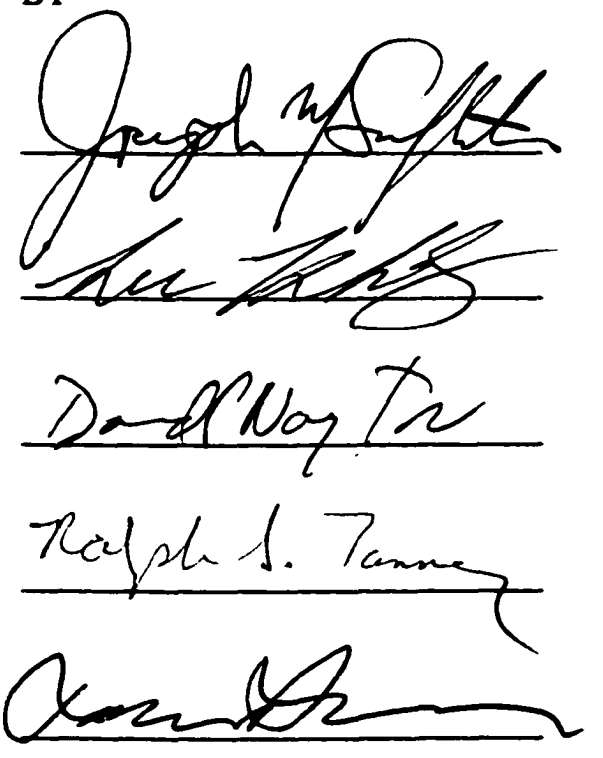
A DISSERTATION APPROVED FOR THE

DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY



Dad,

You gave me the discipline to set goals, the intelligence to accomplish them, and the tenacity to see them through. Then you taught me how to use these attributes.

This dissertation is dedicated to your beloved memory.

Acknowledgements

Although we may try our best, it is not possible to complete work of this breadth without at least some guidance and support along the way. At one time or another I have gone to all members of my committee for both of these things, and would like to thank Dr.'s David Nagle, Ralph Tanner, and Tom Dewers for their help and advice over the last five years. I would also like to acknowledge Dr. Mike McInerney who was extremely helpful in teasing out the pathway found in chapter three.

My fellow graduate students probably helped more day to day than anyone else, since on more occasions than I can remember we tossed ideas around and verified each others' data before going to "the boss". Hence, I have to especially thank Luis Rios-Hernandez, Matt Ramsey, John Senko, and Steve Harris.

My support frame of course comes from my family. My mom and dad encouraged me to follow my goals and have helped in more ways than I can count. In the last couple of years, the one that stuck it out with unwavering support to achieve this degree, good days and bad, was my wife Nicole whom I thank utterly.

Finally, Dr.'s Lee Krumholz and Joe Suflita. I came to OU thinking I knew volumes, and now I do. Although frustrating at times when I thought I had the answer and then heard the infamous "try this and we'll see", I have learned more than you know, both in the lab and out, and I thank-you for that.

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Preface

Radionuclide contamination of the environment is an unfortunate legacy of the 20th century cold war. Uranium in particular has polluted both surface and subsurface sediments and groundwater. This radionuclide is a serious health hazard due to the associated radioactivity and cellular toxicity. While U(VI) is soluble and of primary interest with respect to environmental contamination, U(IV) is insoluble and precipitates, thereby mitigating concerns over the migration of this element in groundwaters. It is well known that microorganisms can catalyze this transformation. In an effort to understand the microbiology of uranium-reduction, this process was studied in the field and with a pure culture bacterium.

This dissertation contains three chapters dealing with various aspects of microbial uranium-reduction. Additionally, Appendix A describes a simple procedure for the enumeration of methanogenic bacteria through the quantitation of coenzyme M. The Appendix only indirectly relates to the main subject of the dissertation. Dr. Ralph Tanner was a contributing author and conducted the most probable number enumerations that facilitated the standardization of the CoM method.

Chapter one was written in the style recognized by the journal *Microbial Ecology* and characterizes a uranium-contaminated site at Shiprock, NM. The goal was to do a microbial and geochemical reconnaissance of the site in an effort to elucidate the important parameters influencing microbial uranium-reduction. This was accomplished by assaying the predominant electron acceptors at the site

and the associated populations of culturable anaerobic bacteria. Metabolic investigations implicated both Fe(III)- and sulfate- reducing bacteria in *in-situ* U-reduction, but only after the exhaustion of nitrate. The addition of exogenous electron donors stimulated the above activities except U(VI) loss, suggesting that electron donors levels do not necessarily limit U-reduction rates. Finally, although high levels of Fe(III) and sulfate were present, these electron acceptors appeared to govern their respective rates of depletion in that a positive correlation was found. Contributing authors for this work were Dr. Philip Long who coordinated sampling efforts at the Shiprock site as well as data organization from various investigators, and Dr. Denny Wong who conducted experiments to test the effect of clays on sulfate-reduction.

Chapter Two was written for the *Journal of Microbiological Methods* and describes a relatively simple method for the quantitation of insoluble U(VI) during bioremediation studies. Although most investigators measure soluble U(VI) and U(IV), a third U pool exists which is apparently not bioavailable until the soluble form becomes exhausted. The procedure did not interfere with quantitation of the other two fractions which found to be the larger of the two U(VI) pools in sedimentary systems. John Senko aided in the assessment of this procedure by developing the method for purification of biologically produced U(IV).

The research presented in Chapter Three deals with the proposed uranium reductase, the periplasmic cytochrome c_3 and was written for *Applied and Environmental Microbiology*. The kinetic parameters of U-reduction in

Desulfovibrio vulgaris Hildenborough were found to agree with previous investigations. However, while U-reduction occurred, it was inhibited by Fe(III), and likewise the presence of U(VI) halted sulfate-reduction. These electron acceptors were used in combination with H_2, lactate, and pyruvate as electron donors in metabolic assays in concert with reduced minus oxidized cytochrome spectra to determine if the path of electron flow from the donors to each acceptor involved this cytochrome. The result is a proposed electron transfer pathway that not only explains the findings presented herein, but also helps clarify several reports in the literature. In brief, the loss of sulfate via electron transfer from lactate or pyruvate involves cytochrome c_3, while H_2-mediated sulfate-reduction does not. Further, the reduction of Fe(III) and U(VI) directly utilizes the cytochrome. Finally, since metal-reduction takes place in the periplasm while sulfate loss occurs in the cytoplasm, this finding offers a hypothesis as to why these microorganisms cannot grow with Fe(III) or U(VI).

Furthering our understanding of the intricacies by which anaerobic bacteria survive in radiologically-contaminated environments is essential to decontaminate these ecosystems. It is my belief that, although this type of pollution is widespread, it can be alleviated using new forms of biotechnology which, in turn, require an extensive understanding of the complex interplay between abiotic and biotic processes.

Abstract

The microbial reduction of uranium was investigated in a contaminated aquifer and with a pure culture model organism, *Desulfovibrio vulgaris* Hildenborough. The concentrations of Fe(III), U(VI), nitrate and sulfate were highly variable within the aquifer, with the latter anion being quantitatively more important than the other potential electron acceptors. While this degree of heterogeneity made it difficult to discern the predominant electron accepting processes that impact U-reduction rates, several trends were notable. Nitrate inhibited the reduction of the other electron acceptors, and once it was biologically removed, the reduction of Fe(III), U(VI), and sulfate occurred concomitantly. The simultaneous loss the latter electron acceptors can be partially explained with the model sulfate reducing bacterium. In *D. vulgaris*, Fe(III) inhibited U-reduction while U(VI) interfered with sulfate loss from organic electron donors. However, during H_2-mediated sulfate-reduction, Fe(III) impeded U-reduction, but only slowed sulfidogenesis, implicating separate electron transfer pathways. Hence, when H_2 served as an electron donor, U(VI) and sulfate could be reduced concomitantly. A second trend noted during field investigations was that the presence of clays inhibited sulfate- but not Fe(III)- or U(VI)-reduction. In sandy sediments, reduction rates for the former two activities showed a positive correlation with their respective acceptor concentrations. This relationship may also be partially explained by the pure culture investigation. With lactate as the donor, 2 mM U(VI) inhibited the reduction of 2 mM sulfate, suggesting preferential electron flow to uranium. However, sulfidogenesis was

only slowed in the presence of 2 mM U(VI) when 20 mM sulfate was used. In the presence of U(VI), high sulfate concentrations may be required to overcome this inhibitory effect on sulfate-reduction. During field investigations it was also found that a considerable amount of the total oxidized uranium present in sedimentary systems was complexed U(VI). When assessing remediative efforts, this pool should also be measured since only soluble U(VI) and U(IV) are routinely quantified, and the complexed pool will most likely be grouped with U(IV), thus underestimating the total U(VI) pool size. Through the development of a new procedure, this fraction of the total uranium pool can be measured without interfering with the quantitation of soluble U(VI) and U(IV). By tracking all three pools during microbial U-reduction, it could be shown that the complexed U(VI) is not initially bioavailable, but appears to solubilize as soluble U(VI) becomes reduced. Hence it is only when both U(VI) pools are reduced can migration into water bodies be considered negligible.

Chapter 1

Characterization of Microbial Activities and U-Reduction in a Shallow Aquifer Contaminated by an UMTRA Disposal Cell

Abstract

A characterization of the Shiprock, NM uranium mill tailing site focused on the geochemical and microbiological factors governing *in-situ* uranium-redox reactions. Groundwater at the site contained a wide range of sulfate, nitrate, Fe(III), and U(VI) concentrations with median values of 9.7 mM, 0.3 mM, 4.8 μM, and 0.4 μM, respectively. Bacterial diversity down gradient from the disposal pile reflected the predominant geochemistry with relatively high numbers of sulfate- and nitrate- reducing microorganisms, and smaller numbers of acetogenic, methanogenic, nitrate-dependent Fe(II)-oxidizing, Fe(III)-reducing, and sulfide-oxidizing microorganisms. In aquifer slurry incubations, nitrate-reduction was always preferred, and negatively impacted sulfate- , Fe(III)- , and U- reduction rates. We also found that sulfate-reduction rates decreased sharply in

the presence of clay, while Fe(III)-reduction increased, with no clear impact on U-reduction. In the absence of clay, iron- and sulfate- reduction correlated with concentrations of Fe(III) and sulfate, respectively. Rates of U(VI) loss did not correlate with the concentration of any electron acceptor. With the exception of Fe(III), electron donor amendment was largely unsuccessful in stimulating electron acceptor loss over a two week incubation period, suggesting that endogenous forms of organic matter were sufficient to support microbial activity. Our findings suggest that efforts to accelerate biological U-reduction should initially focus on stimulating nitrate removal.

Introduction

Contamination of the subsurface with uranium, heavy metals, and radionuclides is of concern to both the environment and to human health. When soluble uranium reaches the subsurface, it migrates with groundwater to potable water bodies and can biomagnify in food chains (14,19,23,49). The reduced form of uranium (U(IV)) is insoluble and of far less environmental concern (2,28,43,50). Microorganisms capable of U-reduction include fermenters like *Clostridium* sp. (16), Fe(III)-reducers such as *Shewanella putrefaciens* (17,31) and *Geobacter metallireducens* (32), the sulfate-reducing bacteria *Desulfovibrio (Dsv.) desulfuricans* (17,31,33,47) and *Desulfotomaculum reducens* (46), and a denitrifying *Pseudomonas* sp. (1,5,15). Thus microbial U-reduction is often considered an important remedial option in contaminated locales.

However, the potential for uranium bioremediation is a function of multiple interacting factors. These include the presence of suitable microorganisms, the availability of electron- acceptors and donors, as well as sediment components that may complex with uranium (36,37). Subsurface microbial communities often exhibit a preference in electron accepting processes when several options are available. For instance, nitrate-reduction tends to predominate (40,43) when available followed by Fe(III)- (11), and then sulfate- reduction (26,34). It is unknown how U-reduction might integrate into such indiscriminate patterns of microbial activity.

The addition of electron donors has been used to differentially stimulate particular microbial respiratory processes. For instance, the use of methanol to stimulate methanogenesis even in the presence of high levels of sulfate has been known for many years (6,25). Knowledge of the electron donors that might favor U-reduction is largely unknown, although a recent report in sediment slurries showed stimulation of both Fe(III)- and U(VI)- reduction, but not sulfate-reduction, through the addition of 100 μM acetate (21). The relevance of such small amendments relative to the large endogenous pool of available electron donors in contaminated areas is at least debatable. However, such treatments can sometimes serve to implicate the kinds of microorganisms that can be stimulated to catalyze U-reduction.

In addition to the aforementioned factors, subsurface strata can impact microbial activity in various ways. Clay layers harbor lower numbers of culturable bacteria than sandy sediments, and generally exhibit lower electron acceptor transformation rates (10,34,48). Clay can also limit the bioavailability of U(VI) (38), via sequestration (D. Elias et al., submitted to Journal of Microbiological Methods).

While the above factors may be evaluated individually, study of their complex interplay at contaminated sites is far more realistic. A Uranium Mill Tailings Remedial Action (UMTRA) site in Shiprock, NM was chosen for this investigation. This site is known to be U-contaminated, and serves as a model for other contaminated Department of Energy lands. The site is a former uranium mill

where wastes associated with U mining and processing were consolidated into a mill tailings disposal pile (http://www.em.doe.gov/bemr96/ship.html).

We characterized the subsurface microbial community and the potential for *in-situ* U-reduction relative to other processes. We also attempted to evaluate the impact of lithology and exogenous electron donor amendments on U-reduction. The study of a real contaminated site helps provide practical insights on the steps needed for U-bioremediation.

Methods

Sample collection

Alluvial floodplain sediments were collected between October, 1998 and April, 2000. Cores were obtained from different depths containing sandy and clay sediments, as well as some Mancos Shale using augers, backhoes, and rotosonic drilling equipment (9,21,22). Groundwater was sampled as previously described (9). All samples were immediately placed under argon and refrigerated at 4°C until used.

Geochemical characteristics

Initial characterization of the Shiprock site began with a measurement of the geochemical parameters that would be important in supporting microbial activity. Analysis of groundwater involved centrifugation (27,000 x g, 20 min, 10°C) to remove solid particles, while sediment porewater was obtained by mixing sediment (1g) with anaerobic water (10 ml), and centrifuging (27,000 x g, 20 min, 10°C). Chloride, nitrate, and sulfate concentrations were determined using a Dionex DX 500 ion chromatograph with an AS4A anion column. Levels of Fe(II)/Fe(III) were determined by the colorimetric method described previously (29).

Samples were prepared for U(VI) analysis by centrifugation of sediments (27,000 x g, 20min, 10°C) and filtering (0.45μm) the pore water supernatant. This was followed by acidification with 10% (v:v) HNO_3 and boiling to dryness. The residue was then resuspended in 1 M HNO_3 to ensure no sequestration or

photoreduction of the uranium (7). The levels of U(VI) were measured using a kinetic phosphorescence analyzer (Model KPA-11, Chemchek Instruments, Inc.) as described previously (7,35).

Bacterial enumerations

To assess the types of bacteria within the subsurface community, a variety of microorganisms were enumerated from sediment samples using a 3-tube most probable number (MPN) assay (45). Sediment from locales directly down gradient of the disposal pile were used to determine which bacterial groups were dominant in this highly contaminated area. All tubes were incubated at room temperature (approximately 20°C) in the dark for 6 weeks before being scored for both growth and loss of electron acceptor. Sulfate- (45) and nitrate- (4) reducing bacteria were enumerated using media previously described. The loss of sulfate and nitrate were determined after 6 weeks by centrifugation (27,000 x g, 20min, 10°C) of a culture medium aliquot followed by ion chromatography as above. Acetogenic bacteria were quantified using media previously described (41) with acetate production determined by gas chromatography. Briefly, samples were diluted 1:20 in 30mM oxalic acid and injected onto a glass Supelco column (2mm x 2m; 80/120 Carbopac B-DA/4% Carbowax 20M) with the injector and detector of a Varian 3400 GC set at 200°C, and the oven set at 155°C. Fe(III)-reducing bacteria were cultured using the described medium (30) with the loss of Fe(III) and consequent increase in Fe(II) determined using the colorimetric assay (29) designed for that purpose. Nitrate-dependent, Fe(II)-oxidizing bacteria were grown (8) and activity verified by determining the loss of Fe(II) and increase in

Fe(III), as well as the loss of nitrate as above. Aerobic Fe(II)-oxidizing bacteria were estimated using a previously described medium (41) with the loss of Fe(II) and increase in Fe(III) determined as above. Sulfide-oxidizing bacteria were enumerated (44) and analyzed for sulfide loss by centrifuging an aliquot of medium followed by quantitation using a colorimetric assay (12). The methanogen population was determined using the Coenzyme M assay (13).

Effect of nitrate on electron accepting activity

When several potential electron acceptors are available in the subsurface, nitrate is preferred (40,43). Hence we tested if this was the case in Shiprock sediments, and what effect the presence of nitrate might have on sulfate- , Fe(III)- , and U(VI)- reduction rates. Slurry incubations contained sediment (20 g) and filter-sterilized groundwater (50 ml) under a N_2/CO_2 atmosphere. These included an autoclaved negative control and a nonsterile sample amended with uranyl-carbonate (2 mM, (33)) to ensure that sufficient U(VI) was available. Electron acceptor concentrations were periodically determined by aseptically removing groundwater (1 ml), centrifuging (22,700 x g, 20 min, 10°C) to remove solid material, and analyzed as above. In addition, the production of sulfide was quantified using a passive extraction procedure (48) and colorimetric assay (12). As previously described, methane was measured periodically by removing 0.2 ml samples of headspace and analyzing by gas chromatography (24). All incubations were at room temperature in the dark and in triplicate.

Effect of clay on electron accepting activity

Sediment cores from various depths were obtained in the drilling operation and used to test the influence of clays on sulfate- , Fe(III)- , and U(VI)- reduction rates. Clays are known to inhibit sulfate-reduction (30,48). Subsamples from the cores were obtained using sterile knives where changes in the sediment lithology (sand vs. clay) were obvious. Incubations were as above except that no exogenous uranium was added. The sediments were sacrificed periodically for electron acceptor measurements as described above.

Effect of electron donor amendments

Slurry incubations were constructed from sediment samples containing low (5 μM) and high (0.3 mM) nitrate levels, and amended with various electron donors. This was done in an effort to stimulate *in-situ* uranium-reduction, and discern if sufficient endogenous electron donor was available to support microbial activity. We equated U(IV) precipitation with the reduction of U(VI) to U(IV) (18). Electron acceptor concentrations were periodically determined as described above. Electron donor concentrations were monitored using an AS-11A column on the Dionex 500 ion chromatograph. The incubations consisted of an autoclaved negative control, sediment amended with 2 mM uranyl-carbonate to quantitate endogenous levels of uranium-reduction, and amendments of 2 mM uranyl-carbonate + 20 mM Na-acetate, Na-formate, Na-lactate, ethanol, or methanol.

Results

Geochemical characteristics

Electron acceptor concentrations in sediment porewater and groundwater spanned extremely large ranges (Fig. 1). Sulfate was quantitatively the dominant electron acceptor with a median concentration of 9.7 mM, while nitrate, Fe(III), and U(VI) concentrations had median values of 4.8 μM, 0.3 mM, and 0.4 μM, respectively (Fig. 1). Nitrate exhibited a wider concentration range relative to other potential electron acceptors. That is, samples ranged from below detection to greater than 134 mM. Not surprisingly, Fe(III) was below detection in groundwater samples given its poor solubility (27), but was up to 1.9 mM in sediment porewaters. The U(VI) concentration ranged from below detection to 42 μM in groundwater and sediment porewaters.

Bacterial Enumerations

This basic geochemical profile was largely consistent with microbial enumeration assays. Interrogation of several samples with depth along the groundwater flowpath revealed that sulfate-reducing bacteria varied from $2.0x10^1$ to 4.3×10^4 cells g^{-1} sediment with comparable numbers for nitrate-reducers ($9.3x10^1$ to 2.3×10^4 cells g^{-1}) (data not shown). Other bacterial types were also found including acetogens (10^3 cells g^{-1}), methanogens (10^3 cells g^{-1}), and both aerobic (4 cells g^{-1}) and anaerobic (10 cells g^{-1}) Fe(II)-oxidizing organisms. The Fe(III)-reducing bacteria in both the groundwater and sediments were either

below the detection limit or at very low values (4 cells g^{-1}). This degree of microbial diversity is not unusual in sedimentary systems and is consistent with previous findings on the importance of sulfate-reducing bacteria (9) at the Shiprock UMTRA site. However, the high degree of variability associated with electron acceptor availability does not preclude the possibility of localized areas where the dominant microbial populations can vary substantially. This point notwithstanding, it is well recognized that the presence and abundance of microorganisms does not necessarily correlate with their *in-situ* activity. Consequently, our efforts were focused on the determination of the predominant electron accepting activity in samples obtained from the site.

Effect of nitrate on electron accepting activity

Even though nitrate was not the most prevalent electron acceptor, in all cases it was preferentially consumed over sulfate, Fe(III), and U(VI). The latter electron acceptors were not rapidly consumed until the former was nearly depleted (data not shown). The impact of nitrate can be seen by a comparison of the sulfate- , Fe(III)- , and U(VI)- reduction rates before and after nitrate consumption in sediment samples (Fig. 2). The sulfate loss rate ranged from 0-2.9 nmol g^{-1} d^{-1} with a median value of 0.2 nmol g^{-1} d^{-1} when nitrate was greater than 5 μM in the incubations (Fig. 2). When nitrate fell below detection limits, sulfate-reduction rates increased to a median of 2.7 nmol g^{-1} d^{-1}. A similar, but less drastic effect was seen for the Fe(III)- and U- reduction rates. These rates increased in the

absence of nitrate from a median value of 1.3 to 4.2 nmol g^{-1} d^{-1} for Fe(III), while the comparable determination for U(VI) was 0.05 to 0.15 pmol g^{-1} d^{-1}.

Effect of clay on electron accepting activity

Of the 40 samples taken from the Shiprock UMTRA site, 24 had a substantial amount of nitrate (above), while 16 had little or no endogenous level of this anion. Without the overwhelming influence of nitrate, the prospect of other environmental factors influencing electron acceptor reduction was considered. Once again, rates of microbial activity varied considerably for sulfate (6.8-112 nmol g^{-1} d^{-1}), Fe(III) (21-81 nmol g^{-1} d^{-1}), and U(VI) (0.02-0.63 nmol g^{-1} d^{-1}) with median values of 21.4, 28.5, and 0.23 nmol g^{-1} d^{-1}, respectively.

These rates were compared with the concentration of the respective electron acceptor in the sample (Fig. 3). We found a correlation between the sulfate concentrations and the rate of sulfate-reduction (Fig 3A) (r^2=0.831). However, this correlation was based on samples that exhibited sulfate-reduction. Seven of the 16 samples exhibited insignificant levels of sulfate loss and each was found to be a predominantly clay-containing sample.

A similar trend was noted for Fe(III)-reduction (r^2=0.839) (Fig. 3B). However, these rates generally increased in clay-containing samples relative to sandy sediments (Fig 3B). In contrast to both sulfate- and Fe(III)- reduction, the relationship between U-reduction and U(VI) concentrations in either the presence or absence of clay was not obvious (Fig. 3C). The range and median value of U(VI) loss was similar in clay samples (0.09-0.55 nmol g^{-1} d^{-1}, median=0.21 nmol

g^{-1} d^{-1}) to those in sandy sediments (0.02-0.63 nmol g^{-1} d^{-1}, median=0.23 nmol g^{-1} d^{-1}).

Effect of electron donor amendments

The relationship between electron acceptor concentrations and their respective reduction processes suggest that the former exerts an important influence on the latter, and that the availability of electron donors is not a limiting factor. To test this hypothesis, we amended sediment slurries with 20 mM acetate, ethanol, formate, lactate, or methanol to sediments that contained high (0.3 mM) and low (5 μM) nitrate concentrations. High performance liquid chromatography (HPLC) analysis revealed that these donors were largely consumed within 16 d. After this period, 3 mM methanol and 2 mM ethanol could be detected in the low nitrate samples. Once again, nitrate was lost preferentially when present (data not shown). There was also a substantial amount of electron acceptor consumption in the donor-unamended controls (Table 1). During the course of this experiment, sulfate was reduced from 9.9 mM to 3.9 mM in the low nitrate samples compared to a decrease from 21.3 mM to 8.3 mM in the high nitrate samples. This represents a 39% reduction in each case. A similar loss was measured for Fe(III)-reduction in the donor-unamended controls in both low (0.68-0.11 mM) and high (0.64-0.45 mM) nitrate sediments. The percentage of U(VI) reduced was greater in low, rather than high nitrate samples even though the initial U(VI) concentration was 160 mM, and is consistent with the inhibitory influence of this anion.

In most cases, the exogenous addition of an electron donor did not significantly stimulate sulfate-reduction over the endogenous controls (Table 1). Notable exceptions to this include lactate and ethanol. The amendments did stimulate Fe(III)-reduction to a statistically significant extent regardless of the initial nitrate concentration in the incubations. However, since iron is a far less quantitatively important electron acceptor, this stimulation could only account for 0.16 to 1.0 mM electron donor consumption. The same electron donor treatments had relatively little influence on U-reduction with the possible exception of methanol. Collectively these findings suggest that the endogenous amounts of electron donors are not limiting in these incubations. Further, the consumption of exogenous forms of organic matter will likely be coupled with the preferential metabolism of nitrate before other electron acceptors are utilized to a substantial degree.

Discussion

Investigation of the geochemical parameters at the Shiprock site showed a substantial variability in several potential electron acceptors including sulfate, nitrate, Fe(III), and U(VI). A comparison of these levels showed that sulfate was by far numerically dominant, followed by the latter three electron acceptors. While median values for all but nitrate are above safe drinking water levels as outlined by the U.S. EPA (http://www.epa.gov/safewater/mcl.html), several locations on the alluvial floodplain contained greater than 800 times the standard for nitrate. While these levels make this area unsuitable as a source of potable water, there is an abundance of electron acceptor available for microbial respiratory processes.

Bacterial enumerations indicated high numbers of nitrate- and sulfate-reducing bacteria that appear to dominate a diverse microbial community. These results are consistent with previous findings at the Shiprock site (20). Further, since these and other types of microorganisms have been implicated in U-reduction (1,5,31,33,38,42,47), *in-situ* microbial U precipitation would appear possible.

An inspection of the reductive processes for each of these electron acceptors and their respective rates revealed that nitrate loss was preferential in each case when nitrate was available. Subsequently, sulfate-, Fe(III)-, and U-reduction occurred concomitantly, which clearly demonstrates the negative impact of nitrate on the latter processes. This effect was verified by substantial increases in

electron acceptor reduction activity after nitrate was exhausted. The favored reduction of this anion has been shown previously (40,43) and agrees with thermodynamic considerations, especially in sulfate-reducing bacteria where U(VI) is favored over sulfate (27). Since ammonium, but no nitric acid was used in the uranium milling process (3), ammonium oxidation would seem to be the likely source of high levels of nitrate contamination at the site (22). We found evidence for both ammonium-oxidizing and nitrate-reducing populations corroborating earlier work (22), and suggest an active cycling of nitrogenous compounds. One consequence of this cycling is that the intermediates of nitrate-reduction could oxidize U(IV) (39), making nitrogenous compound removal critical to ultimate remediation of U contamination at the site.

In the absence of nitrate, sulfate- and Fe(III)- reduction rates were correlated with concentration of their respective electron acceptors. No such relationship could be seen for U. The presence of clay proved to be an interdicting factor in both sulfate and Fe(III) loss by inhibiting sulfate-reducing bacteria. The inhibition of Fe(III)- and sulfate- reduction has been seen previously (30,48). Cessation of the sulfate-reduction may have allowed for the observed increase in Fe(III)-reduction due to a lack of competition for available electron donors.

We tested for the availability of endogenous electron donors and showed overall stimulation of respiratory processes did not occur. Only lactate and ethanol stimulated sulfate-reduction, while the increase in Fe(III) loss with all five electron donors would only account for a minor fraction of the available electron donor at best. Although we attempted to discern if the amendments also increased

nitrate-reduction, the high endogenous rates of this process made this determination impossible. The lack of U-reduction stimulation with excess exogenous, as well as endogenous, electron donors suggests that longer incubation periods will likely be necessary before such an amendment approach is feasible. These results contradict a recent report where Fe(III)- and U(VI)-reduction were differentially stimulated relative to the loss of sulfate (21). However, the latter experiments were performed on samples from a single locale on the floodplain. This is probably an example of an area that differs from the more generalized site characteristics noted here and are expected when such large variability in geochemical and microbiological properties are encountered.

Thus it would seem essential to recognize that nitrate- and sulfate- reducing bacteria are important microorganisms at this site. The dissimilatory sulfite reductase (DSR) gene of *Desulfotomaculum* sp. was found at many areas of the site with a positive correlation between signal strength and the U(VI) concentration (9). In addition, several biomarkers consistent with *Desulfovibrio*-like signals were found in areas of the floodplain where we have previously observed active microbial U-reduction, which could account for the relatively low uranium concentrations (D. Elias, D. Wong, J. Senko, P. E. Long, J. P. McKinley, J. M. Suflita, and L. R. Krumholz, EOS, Trans, Am. Geophys. Union Fall Meet, vol. 81(48), p.F214, 2000).

It would therefore appear that efforts to stimulate *in-situ* U bioremediation should first focus on nitrate removal by either biotic or abiotic mechanisms. As a more favored thermodynamic process, nitrate-reduction should predominate until

it is removed from the contamination area. While there is adequate endogenous electron donor to drive relatively short-term incubations, a longer term approach for *in-situ* treatment would clearly be more appropriate.

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Table 1: Percent of electron acceptor remaining after 16 days

	High nitrate well (300 μM)			Low nitrate well (5 μM)		
Electron-donor	Sulfate reduced	Fe(III) reduced	U(VI) reduced	Sulfate reduced	Fe(III) reduced	U(VI) reduced
Endogenous	61	71	57	61	62	16
Acetate	57	35*	62	50	19*	20
Ethanol	50*	6*	56	7*	38*	22
Formate	58	0*	64	57	39*	18
Lactate	47*	0*	63	41*	0*	21
Methanol	57	0*	74	78	0*	4*

*- a positive statistical difference from unamended sediments using ANOVA analysis ($p<0.05$).

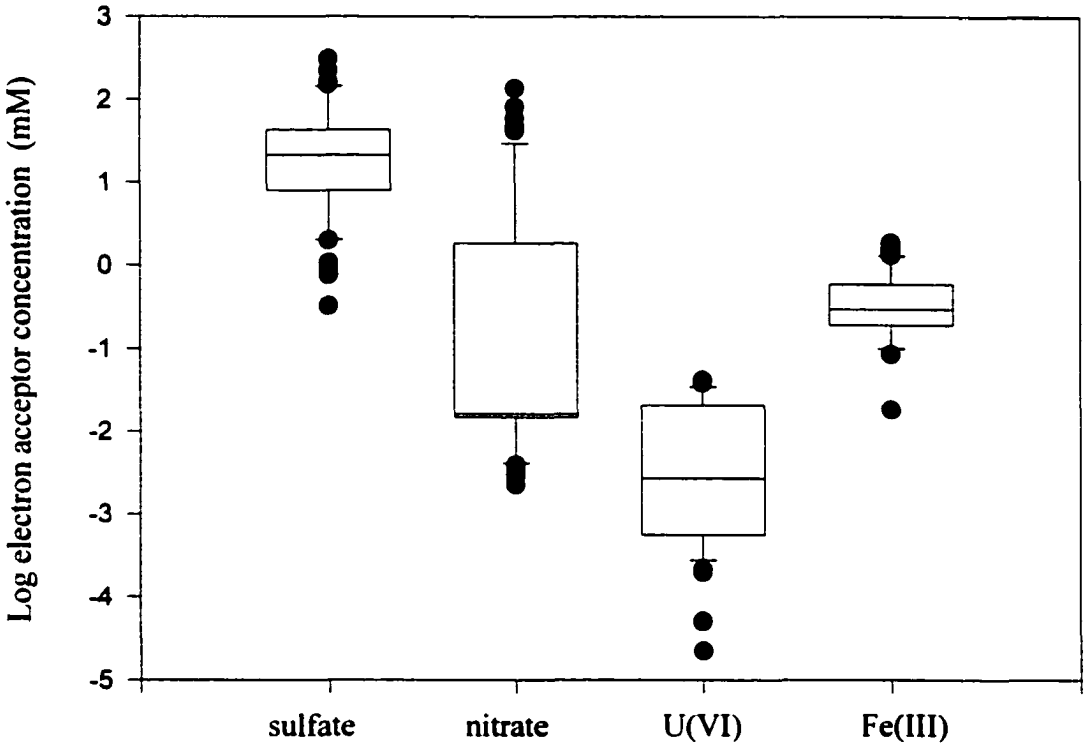

Figure 1: Box and whisker plots for sulfate, nitrate, U(VI) in groundwater and sediment porewater. Levels of Fe(III) are for porewater only. The top and bottom of each box represent the 75th and 25th percentiles, respectively. The middle line of the box represents the median (50th percentile). The whiskers at the top and bottom of the box represent the 10th and 90th percentiles, respectively. The circles represent the 10% lowest and highest electron acceptor concentrations observed.

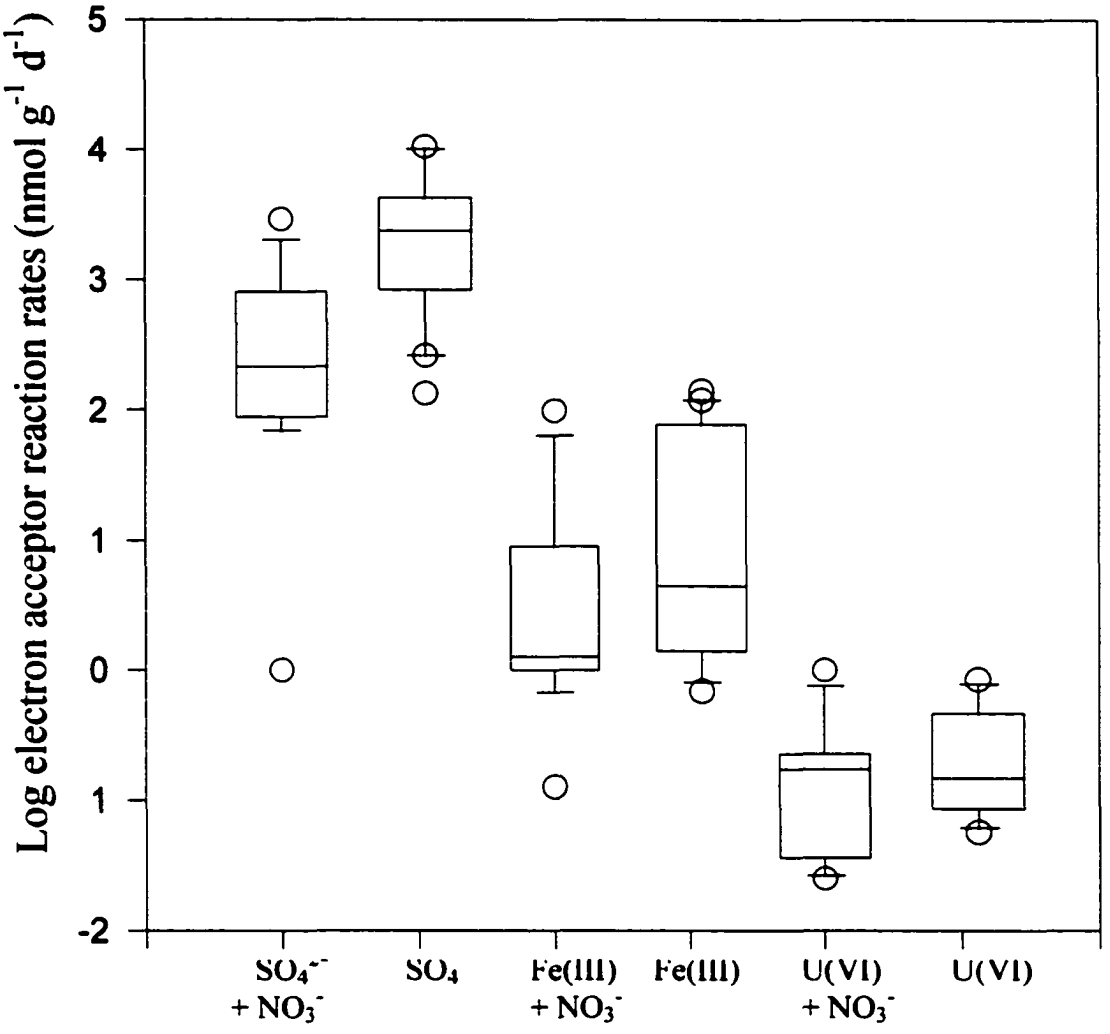

Figure 2: Box and whisker plots for the comparison of sulfate-,Fe(III)- , and U(VI)- reduction rates in the presence of endogenous nitrate concentrations (unfilled boxes) and after nitrate was consumed (shaded boxes).

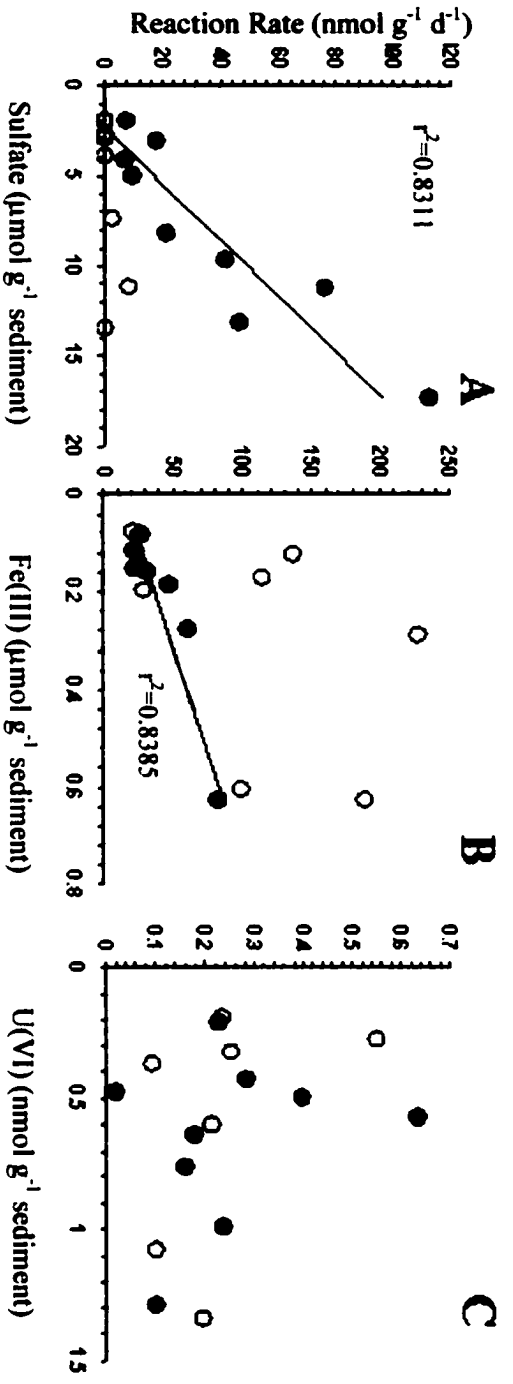

Figure 3: The relationship between electron acceptor concentration and the corresponding reaction rates sediments absent of clay for sulfate (A), Fe(III) (B), and uranium(VI) (C). Samples where clay was present (O) can be distinguished from those where clay was not evident (●).

Chapter 2

A Procedure for Quantitation of Total Oxidized Uranium for Bioremediation Studies

Abstract

A procedure was developed for quantitation of complexed U(VI) during U(VI) immobilization sediment studies. Various extractant volumes to sediment ratios were tested to determine optimal conditions. It was found that from 1:1 to 8:1 (v:w) there was a steady increase in U(VI) recovered, but no change with further increases in volume.

Various strengths of $NaHCO_3$, Na-EDTA, and Na-citrate were used to evaluate complexed U(VI) recovery, while the efficiency of a single, versus repeated extraction steps were compared with synthesized uranyl-phosphate$_{(s)}$ and uranyl-hydroxide$_{(s)}$. Total recovery with 1M $NaHCO_3$ was 95.7% and 97.9% from uranyl-phosphate$_{(s)}$ and uranyl-hydroxide$_{(s)}$, respectively, compared to 80.7% and 89.9% using 450 mM $NaHCO_3$. Performing the procedure once yielded an efficiency of 81.1% and 92.3% for uranyl-phosphate$_{(s)}$ and uranyl-hydroxide$_{(s)}$

respectively, as compared to 3 times. All other extractants yielded 7.9% - 82.0% in both experiments.

Biologically reduced $U(IV)_{(s)}$ was treated either alone or mixed with uncontaminated sediment slurries to ensure the procedure was not interfering with subsequent $U(IV)_{(s)}$ quantitation. While $U(VI)_{(aq)}$ was recovered, it represented 0.07% of the total uranium alone or 7.8% when mixed with sediments. Total uranium recovered did not change.

The procedure was then used to monitor changes in complexed $U(VI)_{(s)}$ levels during U-reduction in pure culture and sediments. There was no appreciable complexed $U(VI)_{(s)}$ concentration in pure culture. In sediments however, once $U(VI)_{(aq)}$ levels and reduction rates decreased, $U(VI)_{(s)}$ levels began to decrease while $U(IV)_{(s)}$ levels continued to increase. This indicated that once $U(VI)_{(aq)}$ was nearly exhausted, sorbed $U(VI)_{(s)}$ became bioavailable and was reduced microbiologically.

Typically, uranium is quantitated in two steps, soluble U(VI) and $U(IV)_{(s)}$. However, the present study shows that after successive washings with water to remove soluble U(VI), a significant pool of oxidized uranium remains that may be mistakenly quantitated as U(IV). This procedure can be used to quantitate this pool, does not interfere with U(IV) quantitation, and has an overall recovery of 95.8%.

Introduction

Contamination of sediments and groundwater with uranium is a global problem. As such, extensive efforts have been put forth to investigate chemical and biological methods of remediating these areas. While a number of reports have shown that chemical methods can clean contaminated sediments and groundwater, these costs are often prohibitive. As such biological methods, primarily microbiological, are seen as a cost effective alternative for clean up of uranium contamination (Barton et al., 1996).

The theory behind U bioremediation is to use the naturally occurring subsurface bacteria to reduce U(VI) which is soluble, to U(IV) which is less soluble/mobile. Further, this ability to reduce U(VI) has been shown in a variety of Fe(III)-reducing (Lovley et al., 1991; Ganesh et al., 1997), and sulfate-reducing (Lovley et al., 1993; Tebo and Obraztsova, 1998; Tucker et al., 1998) bacteria.

We typically quantify U in a contaminated aquifer by measuring the soluble U(VI) concentration in either groundwater or sediment pore water, to arrive at the total U(VI) concentration. This is followed by acidification of the sediments with HNO_3 which oxidizes and solubilizes the remaining U (Brina and Miller, 1992; McKinley et al., 1995) and thus the amount of U(VI) relative to the total amount of U contamination can be assessed.

However, an aspect of this process commonly overlooked is that once soluble U(VI) is depleted, it is assumed that all remaining uranium is U(IV). This is not necessarily the case. There are in fact 2 separate U(VI) pools in the environment.

The first of these is the aforementioned soluble U(VI), while the second is a complexed $U(VI)_{(s)}$ pool (Langmuir, 1997). The form of the latter is primarily determined by the pH and mineral components of a given system. In pH neutral systems, the dominant mineral forms will most likely be uranyl- $hydroxide_{(s)}$ (Langmuir, 1997) and $phosphate_{(s)}$ (Barten, 1988; Mathur, 1991) (if available), whereas in alkaline systems, hydroxide complexation would dominate (Wahlgren et al., 1999). Association with both anions is thermodynamically favorable, and causes precipitation (Ganesh et al., 1995; Robinson et al., 1998) as indicated by their low solubility in an aqueous system (Langmuir, 1997). Thus, they would not be included in the quantitation of the soluble U(VI) pool.

Another consideration is that at a circumneutral pH, the uranyl ion (UO_2^{2+}) will readily form complexes with shale rock (Arnold et al., 1998; Mossman, 1999), and ligands such as clays (McKinley, et al., 1995), silica and goethite (Gabriel et al., 1998), as well as iron-oxyhydroxides (Charlet et al., 1998). This is further complicated by adsorbance to the *in-situ* microbial population (Francis et al., 1991; Panak et al., 1999). In short, UO_2^{2+} will complex with any number of ligands present in the subsurface, thereby reducing the soluble U(VI) pool, and increasing the complexed $U(VI)_{(s)}$ pool.

Historically, the strategy for extraction of soluble U(VI) has been to complex the uranyl ion with a favorable ligand. Several reports have shown the effectiveness for removal of U(VI) from contaminated sites (Lai and Kao, 1971; Fujinaga and Lee, 1977; Ganesh, et al., 1995) and from human tissue after exposure (Ramanujam et al., 1983; Durbin et al., 1994; Ubios et al., 1994; Durbin

et al., 1997; Durbin et al., 1998; Henge-Napoli et al., 1998; Henge-Napoli et al., 1999; Singh and Gupta, 1999). The most effective chemicals for environmental systems appear to be citrate at a concentration of 2 mM (Francis et al., 1992; Francis and Dodge, 1993; Dodge and Francis, 1994; Dodge and Francis, 1997; Huang et al., 1998) and bicarbonate at approximately 100 mM (Abdelouas et al., 1998); (Phillips et al., 1995). Various chemical ligands (eg. EDTA) are somewhat less effective at approximately 1 mM (Lai and Kao, 1971; Fujinaga and Lee, 1977; Ganesh, et al., 1995). However in this work, we tested these chemicals for the reverse effect, to overcome naturally formed ligands and allow for quantitation of the complexed $U(VI)_{(s)}$ pool.

Previously, it was reported (Phillips, et al., 1995) that 100 mM $NaHCO_3$, pH 8.4 was effective for solubilizing 20-94% of the complexed $U(VI)_{(s)}$ pool during U(VI)-reduction studies. Although the report used sediments from several U-contaminated sites, the range of recovery indicates that a better developed procedure may improve reproducibility and extent of retrieval.

The present work reports a standardized procedure which can quantitate the complexed $U(VI)_{(s)}$ pool using 1M $NaHCO_3$, pH 8.3 with an overall efficiency of 95.8% after a single extraction. The procedure shows that by controlling buffer strength and the ratio of volume to sediment weight, this pool can be effectively and efficiently assessed. This method does not interfere with soluble U(VI) measurement or extract U(IV).

Methods

Determination of optimal extraction volume to sediment weight ratio

In order to determine the optimal extractant volume:sediment weight ratio on U-contaminated sand and clay sediments, 4 different sediments were obtained. Fine and coarse sand, and clay were used from the uranium contaminated Shiprock, NM site. A clay sample from the uranium contaminated Oak Ridge, TN site was also used. Sediments (20 g) were placed in sterile 120 ml serum bottles under an N_2/CO_2 atmosphere, and incubated (1 d). Aliquots (1 g) were placed in 50ml centrifuge tubes and the sediments centrifuged (27,000 x g, 20 min, 20°C) to remove soluble U(VI). Following removal of the supernatant, volumes of 1,2,4,6,8, or 10 ml of 222 mM $NaHCO_3$ (pH 8.3, N_2/CO_2) were added to the centrifuge tubes and left in the anaerobic chamber in the dark overnight. The tubes were centrifuged (27,000 x g, 20 min, 20°C), and the supernatant saved and analyzed using a KPA-10 (Chemchek Instruments, Richland, WA) as previously described (Brina and Miller, 1992). The procedure was repeated 2 more times, and was also tested with 1 mM Na-EDTA and 2 mM Na-citrate using volumes of 2,4,6, or 8 ml/g sediment.

Determination of the Extraction Efficiency using Uranyl-phosphate and Uranyl-hydroxide

To determine the extraction efficiency on a known amount of complexed $U(VI)_{(s)}$, uranyl-phosphate and uranyl-hydroxide were synthesized. Uranyl-

phosphate was made by mixing 20 ml of a 12.5 mM uranyl-acetate stock solution with 0.06 g of Na_2HPO_4 (20 mM HPO_4^{2-}) and stored under N_2. After incubation (24 h, 20°C) in the anaerobic chamber, there was a pale yellow precipitate. The precipitate was washed twice with anaerobic 20 mM $NaHPO_4$ (pH 9.2) by resuspension and centrifugation (27,000 x g, 20 min, 20°C) and stored under N_2 in the anaerobic chamber in 20 ml of 20 mM $NaHPO_4$. After shaking, 0.4 ml was mixed with uncontaminated sediment (20 g) from a leachate-impacted aquifer (Beeman and Suflita, 1987; Beeman and Suflita, 1990; Adrian et al., 1994), and allowed to incubate anaerobically (24 h) at room temperature.

Uranyl-hydroxide was synthesized by first dissolving uranyl-acetate to attain 50 ml of a 10 mM solution and increasing the pH to 10.0 with NaOH. Immediately a yellow precipitate formed. The suspension was centrifuged (27,000 x g, 20 min, 20°C) to remove the supernatant containing soluble U(VI). The pellet was washed twice with 100 mM NaOH and stored under N_2 in the anaerobic chamber in 20 ml of 100 mM NaOH. Once shaken, 0.7 ml was mixed with uncontaminated sediment (20 g) and allowed to incubate anaerobically (24 h).

Solubilization of both uranyl-hydroxide$_{(s)}$ and uranyl-phosphate$_{(s)}$ was performed by aliquoting U(VI)$_{(s)}$ amended sediment (1g) to separate tubes to test each extractant solution. The sediments were washed twice with sterile anoxic water, treated with 8 ml of 30, 50, 100, 150, 200, 222, 450, 1000 mM $NaHCO_3$, or 1,2 mM Na-citrate, or 0.5, 1 mM Na-EDTA, and left in the anaerobic chamber in the dark overnight. All solutions were adjusted to pH 8.3 under a N_2/CO_2 headspace. The tubes were centrifuged (27,000 x g, 20 min, 20°C), and the

supernatant saved and analyzed using a KPA-10. The extraction procedure was performed 3 times in total.

Finally, all tubes were treated with 1 ml of concentrated HNO_3 to quantitate any remaining U that may have been reduced in the sediments (Brina and Miller, 1992). To determine the carryover volume of liquid between extractions, 1 g of sediment was dispensed to an Eppendorf tube, weighed, dried, and weighed again. The difference in weight was used as the volume of liquid (in ml) associated with 1 g of sediment.

To establish the amount of uranyl- phosphate or hydroxide in solutions, an eppendorf tube was weighed, 1 ml of each solution dispensed to the tube, centrifuged (13,000 x g, 10 min, 20°C), the supernatant discarded, and the pellet dried. Once dry, the tube was weighed to determine the amount of U mineral in each solution.

Procedure for purification of biologically reduced U(IV)

We determined whether the extraction procedure oxidized $U(IV)_{(s)}$ by using biologically reduced U prepared with the following procedure. A 1L culture of *Desulfovibrio desulfuricans* Essex6 was grown in the medium previously described (Odom and Wall, 1987). The cells were harvested in an anaerobic chamber and centrifuged (27,000 x g, 20 min, 20°C). The pellet was washed 3 times with 30 mM $NaHCO_3$ buffer, pH 6.8. Cells were resuspended in 10 ml of buffer and injected via syringe into a 160 ml sterile serum bottle (under N_2/CO_2) containing 100 ml of 30 mM $NaHCO_3$ buffer (pH 6.8) amended with 5 mM

uranyl-acetate$_{(s)}$ and 20 mM Na-lactate and incubated (48 h). At that point, the bottle contents were black and settled, and were centrifuged (27,000 x g, 20 min, 20°C) and washed 3 times with 30 mM $NaHCO_3$ buffer, pH 6.8 to remove any $U(VI)_{(s)}$ loosely associated with cell surfaces. The pellet was then resuspended in 1 M NaOH and allowed to incubate (1 h) to dissolve cell membranes and proteins. The suspension was centrifuged again, and the pellet washed in 1 M NaOH 3 times. The pellet was washed with water twice to remove the remaining NaOH, then with 1 M $NaHCO_3$ to remove any complexed $U(VI)_{(s)}$. This was repeated until no $U(VI)_{(aq)}$ could be detected. On average, this required 4 washes. The resulting pellet was finally washed twice in Nanopure water to remove any remaining $NaHCO_3$ and autoclaved (20 min). The resulting solid was greater than 99% $U(IV)_{(s)}$ as determined by an absence of $U(VI)_{(aq)}$ using KPA analysis of the final wash water.

Determination of U(IV) oxidation using biologically reduced U

The biologically reduced $U(IV)_{(s)}$ was used to determine if the appropriate strength of $NaHCO_3$ extractant solution would oxidize U(IV). To accomplish this, 1 ml (16 mg; dry weight) of U(IV) was added to 1.5 ml Eppendorf tubes and centrifuged (13,000 x g, 10 min, 20°C) to remove the water. Solutions of 222 mM or 1 M $NaHCO_3$, 1 mM Na-EDTA, or 2 mM Na-citrate were added (0.16 ml) and anaerobically incubated overnight. The mixture was centrifuged (13,000 x g, 10 min, 20°C) and any $U(VI)_{(aq)}$ quantitated as described above. The procedure was

repeated twice. The remaining $U(IV)_{(s)}$ was oxidized with HNO_3 (1 ml, 4 h incubation) and the resulting U(VI) quantitated.

In order to determine the influence of sediments on the extraction procedure, 1 ml (10 mg) of biologically reduced $U(IV)_{(s)}$ was mixed with sediment (40 g) from an uncontaminated leachate impacted aquifer. Unlike the experiment using U(IV) alone, a significantly smaller amount of $U(IV)_{(s)}$ was used in order to better imitate concentrations found at U-contaminated sites. The incubation was prepared anaerobically using an N_2/CO_2 headspace. This was allowed to incubate in the dark (24h). The sediments were then aliquoted (1 g) to centrifuge tubes, extracted and analyzed as above. The extraction procedure was repeated 3 times. The remaining $U(IV)_{(s)}$ was oxidized with HNO_3 (1 ml, 4 h incubation) and the resulting $U(VI)_{(aq)}$ quantitated.

Measurement of complexed uranium(VI) during microbial uranium-reduction experiments

The quantitation of complexed $U(VI)_{(s)}$ was tested in both a pure culture of *D. desulfuricans* Essex6 and in the uncontaminated sediments. All steps of the procedure were performed in an anaerobic chamber except for U quantitation. The culture was grown and cells washed as described above. Samples from the U-reduction assay (0.2 ml) were removed every 20 min and preserved in 0.2 ml of 1.2 M HCl. The samples were centrifuged (13,000 x g, 20 min, 20°C) and the supernatant removed for soluble U(VI) quantitation. The pellet was resuspended in 1 M $NaHCO_3$ (1.5 ml), incubated overnight, centrifuged, and the supernatant

removed for complexed $U(VI)_{(aq)}$ analysis. The pellet was subjected to 1 ml of 10% HNO_3 (2 h) and the remaining U quantitated was taken as U(IV).

The sediments (1 g) were placed in sterile 25 ml serum bottles and spiked with 100 µl of 1.3 mM uranyl-carbonate. The uranyl-carbonate was prepared according to the method of Phillips (1995). The bottles were sealed with rubber stoppers and flushed with N_2/CO_2.

Measurement of U levels over time was accomplished by periodically sacrificing bottles. Soluble U(VI) was quantitated by removing all liquid possible via pipetter. Changes in complexed $U(VI)_{(s)}$ were monitored by the addition of sterile 1 M $NaHCO_3$, pH 8.3 (8 ml) and incubation overnight. The 1 M $NaHCO_3$ was kept under a N_2/CO_2 atmosphere to eliminate the loss of HCO_3^-/CO_2. After incubation, the $NaHCO_3$ was removed via pipetter and 1 ml of HNO_3 was added to quantify all remaining uranium (Brina and Miller, 1992).

Results

Determination of optimal extraction volume to sediment weight ratio

Determination of the optimal buffer volume to sediment weight ratio was consistent among the four sediments tested (Fig. 1). With fine sand (Fig. 1A), more $U(VI)_{(s)}$ was solubilized with each increase in volume from 1:1 to 10:1 (v:w). However, coarse sand (Fig. 1B) and both clay samples (Fig. 1C, 1D) gave the greatest yields at 6-8:1 ratio with no increase at 10:1 ratio. When expressed as a percent value from the 3 extractions performed, approximately 70% of the U(VI) was present in the first extraction.

Similar results were seen when 2 mM Na-citrate or 1 mM Na-EDTA were used on the fine and coarse sand. The Na-EDTA was not effective for solubilizing complexed $U(VI)_{(s)}$ from either of the clay samples (data not shown).

Extraction of uranyl-phosphate & uranyl-hydroxide

The recovery of $U(VI)_{(aq)}$ from uranyl-phosphate amended sediments using $NaHCO_3$ solutions increased with increasing $NaHCO_3$ concentrations (Fig. 2A). However, neither Na-EDTA nor Na-Citrate solutions showed the same trend. For each extractant buffer, between 96.1-99.9% of the $U(VI)_{(aq)}$ quantitated in repeated extractions was present in the first round. In order to calculate U(VI) recovery from sediments, the amount found in water washes was subtracted from the amount of U(VI) added to the sediments. Therefore, of the total uranium remaining after soluble U(VI) removal (23.7 μg U(VI)/g sediment added to the

system – 4.6 μg soluble U(VI)/g sediment = 19.1 μg U(VI) bound/g sediment), 17.8 μg/g sediment (93.2%) was extracted with 1 M $NaHCO_3$ in the first round compared to 16.3 μg/g sediment (85.3%) and 15.7 μg/g sediment (82.2%) with 222 and 450 mM $NaHCO_3$, respectively (Fig. 2B). The total amount of uranium quantitated from each sample (washes and all extractions) was calculated to be 21.3 – 23.2 μg/g sediment, indicating that each aliquoted sample contained approximately the same amount of uranium.

The 1 ml uranyl-phosphate sample that was dried weighed 3.8 mg, and contained 1.18 mg U(VI), based on HNO_3 extraction (23.7 μg/g sediment). The total recovery from the sediments was 89.8 – 97.9% of the U(VI) added as uranyl-phosphate (Fig. 2B).

Similar results were observed for uranyl-hydroxide. Excluding the water control, 90.5 – 97.6% of the U(VI) recovered in the triplicate extractions was found in the first round (Fig. 2C). However, unlike the uranyl-phosphate, a significant proportion of the $U(VI)_{(aq)}$ was found in the water washes before extraction (data not shown). This would presumably be due to the drop in pH from 10.0 to approximately 6.8 - 7.0 in the sediments. This drop in pH, and the presence of carbonates in the sediment would likely dissolve $U(VI)_{(s)}$. When the $U(VI)_{(aq)}$ quantitated in water washes was subtracted from the amount of $U(VI)_{(s)}$ added to the system (73.5 μg/g sediment), 1 M $NaHCO_3$ extracted 94.3% of the total recoverable $U(VI)_{(s)}$ in the first extraction. As seen with uranyl-phosphate, 222 and 450 mM $NaHCO_3$ recovered less at 82.0 & 89.9%, respectively, while lower concentrations contained less than 80% (Fig. 2D). The recovery of $U(VI)_{(s)}$

in the first extraction with Na-EDTA (20.3%) or Na-citrate (33.4%) were far lower than with 1 M $NaHCO_3$ (Fig.2D).

The dried 1 ml uranyl-hydroxide sample weighed 3.7 mg, and contained 2.1 mg U(VI), (73.5 μg/g sediment). The total recovery from the sediments was 84.7 – 99.3% of the uranium added as uranyl-hydroxide (Fig. 2D).

Determination of U(IV) oxidation during extraction

Tests with biologically reduced U(IV) alone showed that when 1 M $NaHCO_3$ was used, 6.1 μg of $U(VI)_{(s)}$ was removed during the first round, with a total of 7.27 μg found in three extractions combined (Fig. 3). The HNO_3 treatment yielded 1.4 mg of uranium. The U found after one 1 M $NaHCO_3$ treatment represented 0.05% of the total uranium recovered, and this increased to only 0.06% after three 1 M $NaHCO_3$ treatments. Although it is not likely that the $U(VI)_{(aq)}$ recovered in this first step was due to oxidation, it is clear that only a small fraction of the U was oxidized during the whole procedure.

The treatment of biologically reduced U(IV) added to uncontaminated sediment showed results similar to that of the U(IV) alone. $U(VI)_{(s)}$ was more efficiently extracted using 1 M HCO_3 than all other solutions tested (Fig. 4A). Further, based on the 3 extractions performed, the 1 M HCO_3 solution was the only one to solubilize greater than 90% of the U(VI) during the first round (Fig. 4B). Oxidation of the U(IV) does not appear to be important even in the sediments. A total of 7.8% of the biologically reduced U(IV) added was quantitated with 1 M $NaHCO_3$, and up to 6.1% was quantitated using all other

solutions. This could also be explained by the presence of other oxidants in the sediment. We also tested whether the presence of 1 M $NaHCO_3$ would interfere with the HNO_3 used to quantitate the remaining U. There did not appear to be any interference. A total of 10 mg U(IV) (based on dry weight) was added to sediment (40 g). The U(IV) was resuspended in 1 ml of anaerobic water and injected into sediments. A HNO_3 extraction of the sediment before U(IV) addition showed no detectable U present. The same treatment after addition of the U(IV) to the sediments quantitated 8.9 mg U (223.7 μg/g sediment) (Fig. 4C).

Measurement of uranium(VI) during microbial uranium-reduction

The optimized procedure was utilized to measure complexed $U(VI)_{(s)}$ during microbial U-reduction experiments with both *D. desulfuricans* and sediments. The assessment of complexed $U(VI)_{(s)}$ in the pure culture (Fig. 5A) showed that even at 2 mM uranyl-carbonate, a negligible amount of the U(VI) was found as complexed $U(VI)_{(s)}$.

The concentrations of soluble and complexed $U(VI)_{(s)}$ were followed during U-reduction in sediments and we determined that both forms could be accurately followed over time. Initially 30.9 μg of uranyl-carbonate was added to 1 g of uncontaminated sediment. The total uranium quantitated did not change appreciably over the time course (29.82 – 32.68 μg U/g sediment) (Fig. 5B).

Immediately, a substantial loss of soluble U(VI) was observed without an appreciable increase in U(IV) levels. Quantitation of the 1 M $NaHCO_3$-extractable fraction (single treatment) showed that 19.02 of the 30.9 μg U(VI)/g sediment

added (61.6%) had formed $U(VI)_{(s)}$ complexes. As soluble U(VI) concentrations decreased over several days, levels of complexed $U(VI)_{(s)}$ did not change substantially, while HNO_3 extractable U (assumed to be U(IV)) increased. After 4 d, rates of soluble U(VI) loss decreased to negligible levels, and from days 4 – 11, complexed $U(VI)_{(s)}$ concentrations decreased from 16.7 – 3.3 μg/g sediment while U(IV) levels continued to increase.

Discussion

The procedure outlined in this work has shown that the complexed $U(VI)_{(s)}$ pool is a substantial fraction of total U(VI) in sedimentary systems. While the protocol may be used for geochemical determination, we present it as an effective tool for the assessment of bioremediative efforts with uranium. This technique will allow the investigator to follow the biogeochemical changes in total U(VI) levels during microbial uranium-reduction.

Numerous reports of contaminated sites supply data for soluble U(VI) and U(IV) (Lovley and Phillips, 1992; Lovley, et al., 1993; Duff et al., 1999), however virtually none provide data on the size of the complexed $U(VI)_{(s)}$ pool, presumably including it in the HNO_3 extractable (U(IV)) uranium pool. This will seriously underestimate total U(VI) and overestimate the U(IV) pools, resulting in an underestimation of site remediation time. Once the soluble U(VI) concentrations proceed to near zero, the complexed forms of $U(VI)_{(s)}$ should begin to solubilize to equilibrium according to our data, and be reduced and thus immobilized. This may maintain a soluble U(VI) concentration above Environmental Protection Agency standards (30μg/L) (http://www.epa.gov/safewater/standard/pp/radnucpp.html).

To this end, measurement of all 3 uranium pools (soluble U(VI), complexed $U(VI)_{(s)}$, and HNO_3 extractable U(IV)) is required given that most subsurface systems contain abundant sources of complexing agents (Finch and Murakami, 1999). Further, once soluble U(VI) levels are shown to decrease at the site of interest via microbial U-reduction, analysis should shift to monitoring the

decrease in complexed $U(VI)_{(s)}$ levels. The present study shows that only when this pool is exhausted can the threat of oxidized U contamination to ground waters be deemed negligible.

The final HNO_3 extraction is intended to dissolve U(IV) through oxidation. All of the experiments reported here support the assertion that the HNO_3 extractable fraction is mainly U(IV) (Senko et al., 2002).

We have standardized the parameters which are critical for the efficient quantitation of complexed $U(VI)_{(s)}$. It has been shown that 1 M $NaHCO_3$, pH 8.3 is the most effective extractant for assessment of the complexed $U(VI)_{(s)}$ pool without interfering with U(IV) quantitation. Although chelating agents and Na-citrate are effective in releasing $U(VI)_{(s)}$ from insoluble complexes (Lai and Kao, 1971; Fujinaga and Lee, 1977; Francis, et al., 1992; Ganesh, et al., 1995), they are less effective on several sediment types than 1 M $NaHCO_3$.

The parameters for extraction included establishment of the optimal extractant:sediment ratio (v:w) followed by the determination of the strength of extractant to be used. A previous report showed that 100 mM $NaHCO_3$ could extract complexed $U(VI)_{(s)}$, but the efficiency ranged from 20-94% depending on sediment type (Phillips, et al., 1995). In contrast, the present study indicates that 100 mM $NaHCO_3$ was less than 30% as efficient as 1 M $NaHCO_3$ in extracting U(VI) from the synthesized uranyl-phosphate, and approximately 60% as efficient for uranyl-hydroxide. Further, the extractant:sediment ratio (v:w) in the previous report was 5:1 which has been shown here to be approximately 75% as effective as the 8:1 ratio used in this study.

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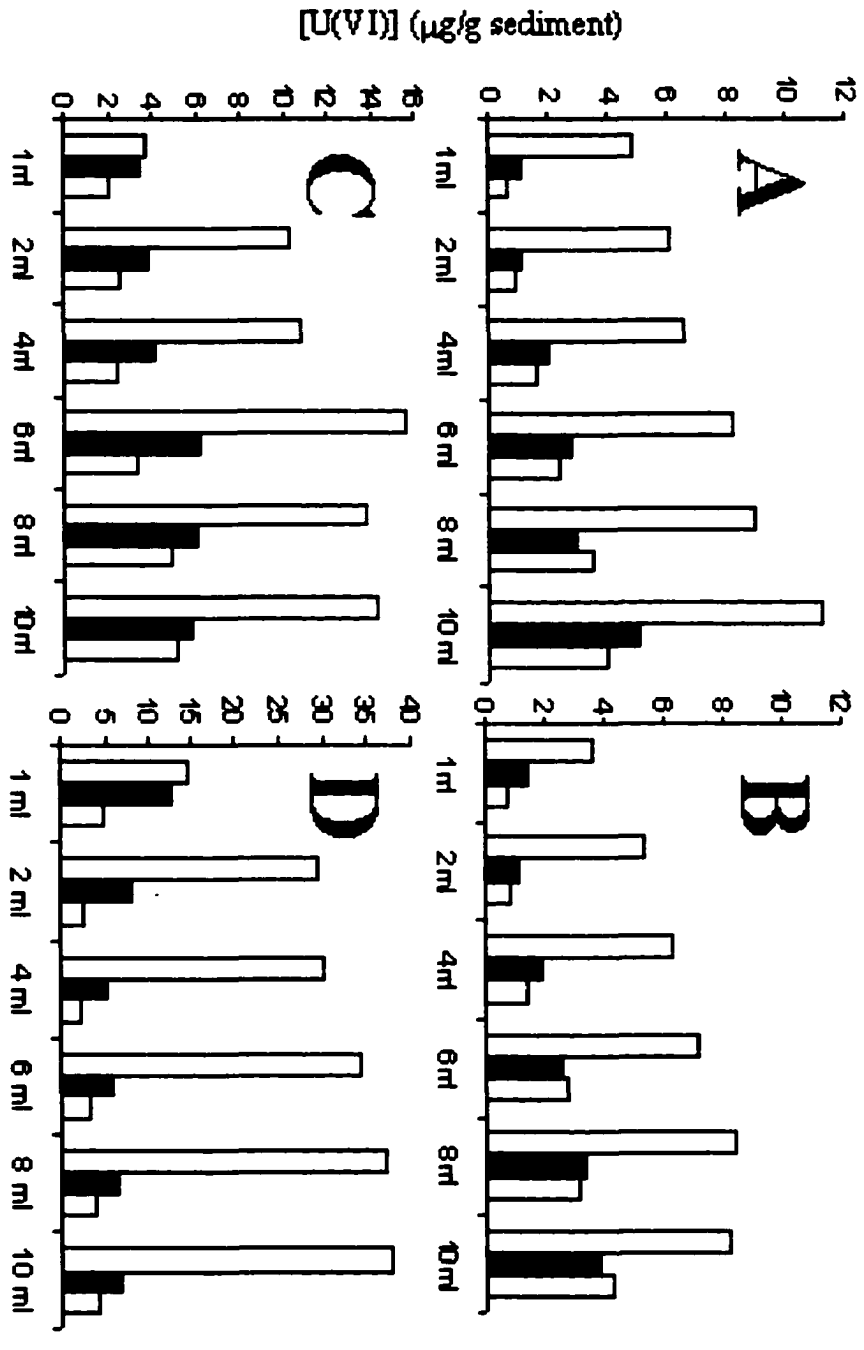


Figure 1: The effect of increasing volumes of 222 mM $NaHCO_3$ on complexed U(VI) quantitation from uranium-contaminated sediments. Three sequential extractions of 1g of (A) fine sand, (B) coarse sand, (C) weathered Mancos shale from Shiprock, N.M. and (D) clay from Oak Ridge, T.N. were performed.

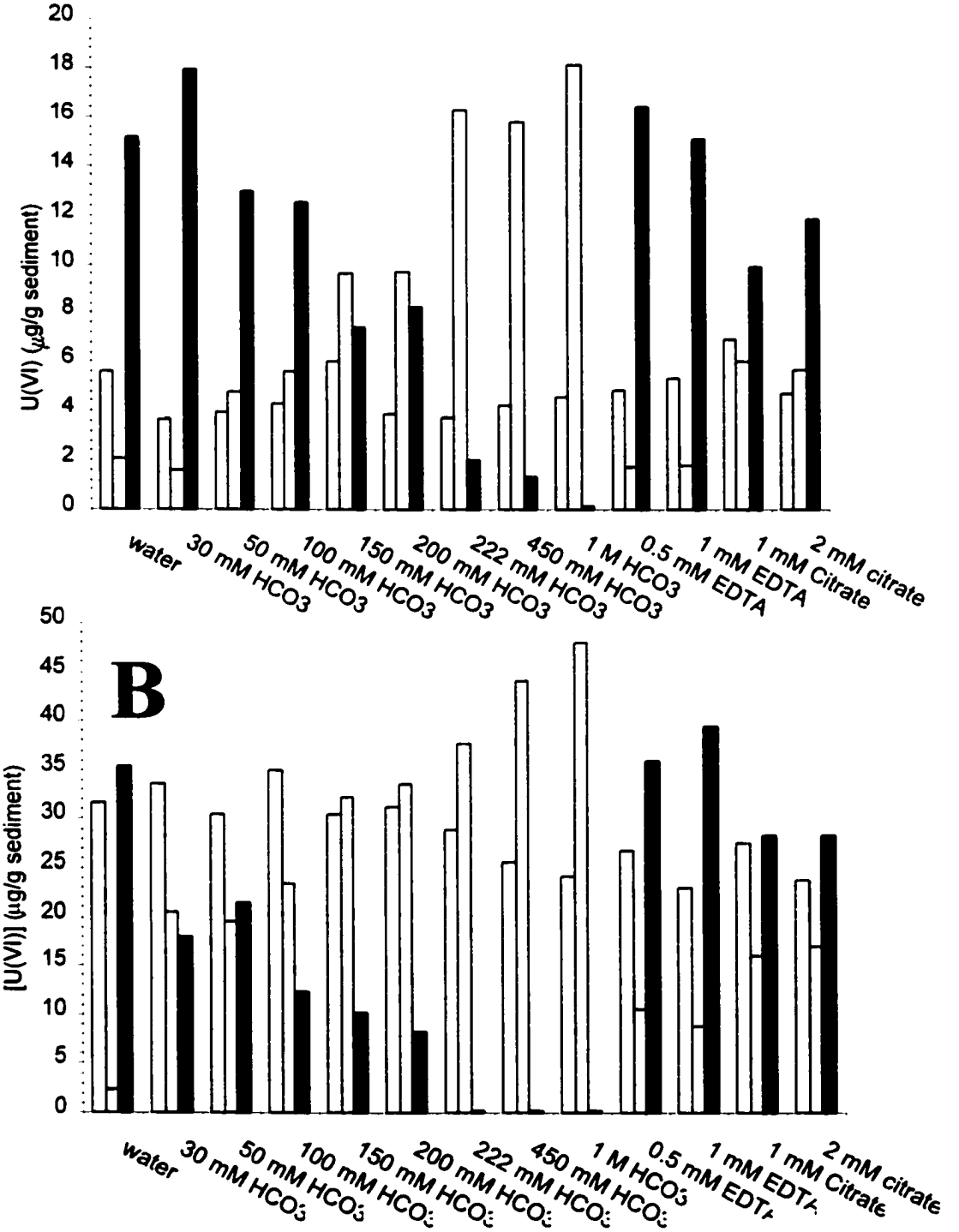
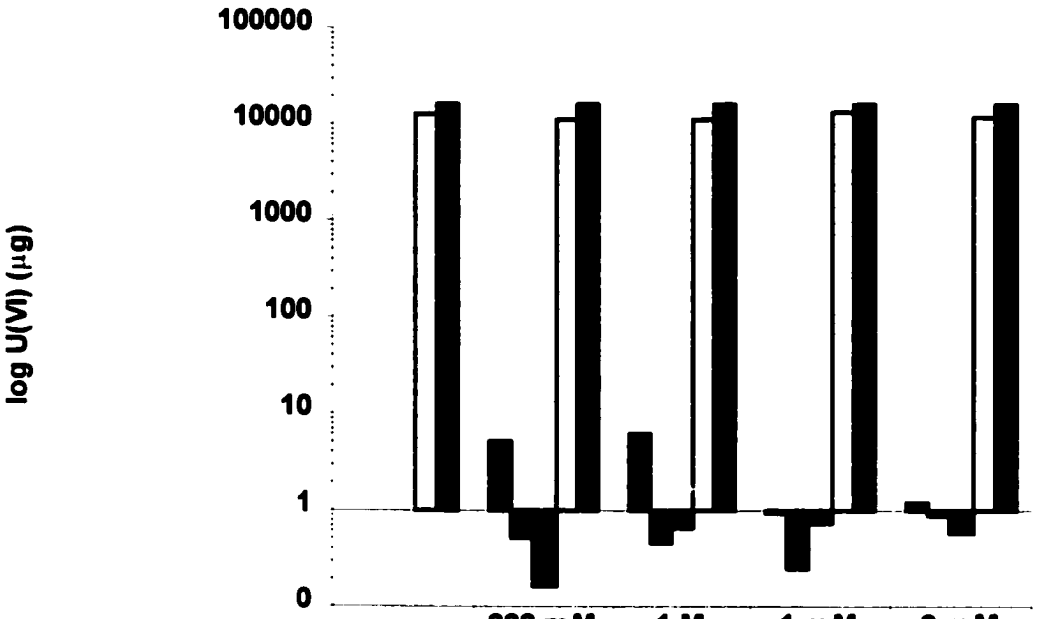


Figure 2: A comparison of the stepwise quantitation of soluble and complexed U(VI), and U(IV) for uranyl-phosphate (A) and uranyl-hydroxide (B)when added to uncontaminated sediments. Each sediment was tested in duplicate.



	water	222 mM HCO3	1 M HCO3	1 mM EDTA	2 mM Citrate
1st extraction	0.00	5.01	6.15	0.95	1.19
2nd extraction	0.00	0.50	0.46	0.25	0.91
3rd extraction	0.00	0.16	0.66	0.74	0.60
HNO3 extraction	12987.24	11275.01	11454.04	13433.48	12176.01
U(iV) added	16000.00	16000.00	16000.00	16000.00	16000.00

Figure 3: Extraction of biologically reduced U(IV) alone with various ligands followed by HNO_3 extraction.

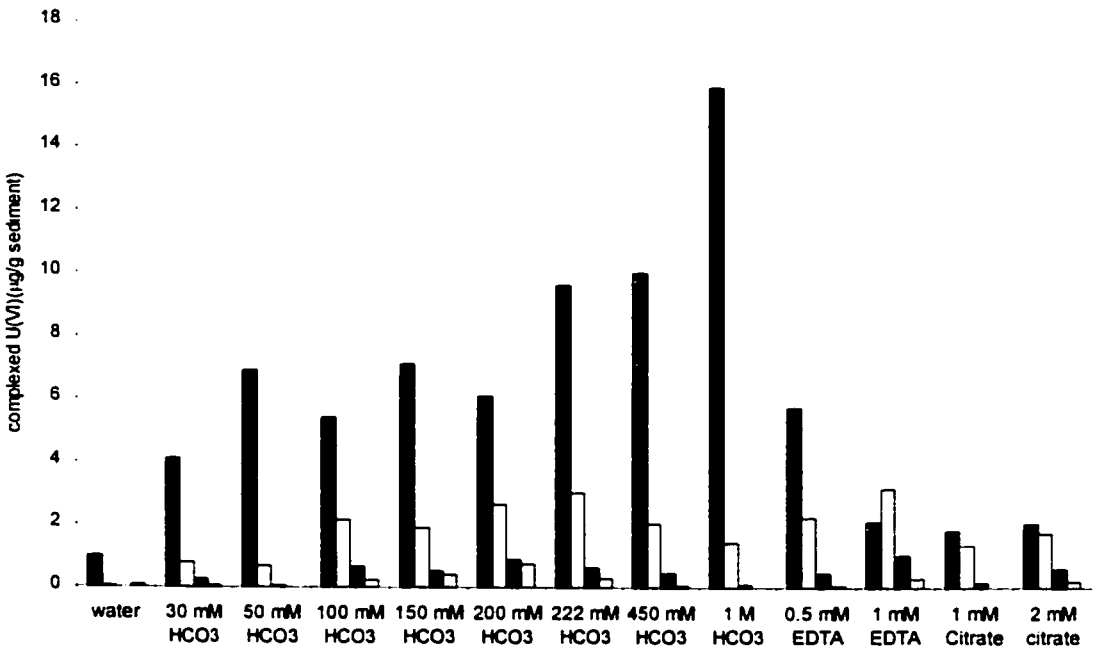


Figure 4: Four sequential extractions of sediment aliquots containing biologically reduced U(IV) to test the utility of various ligands.

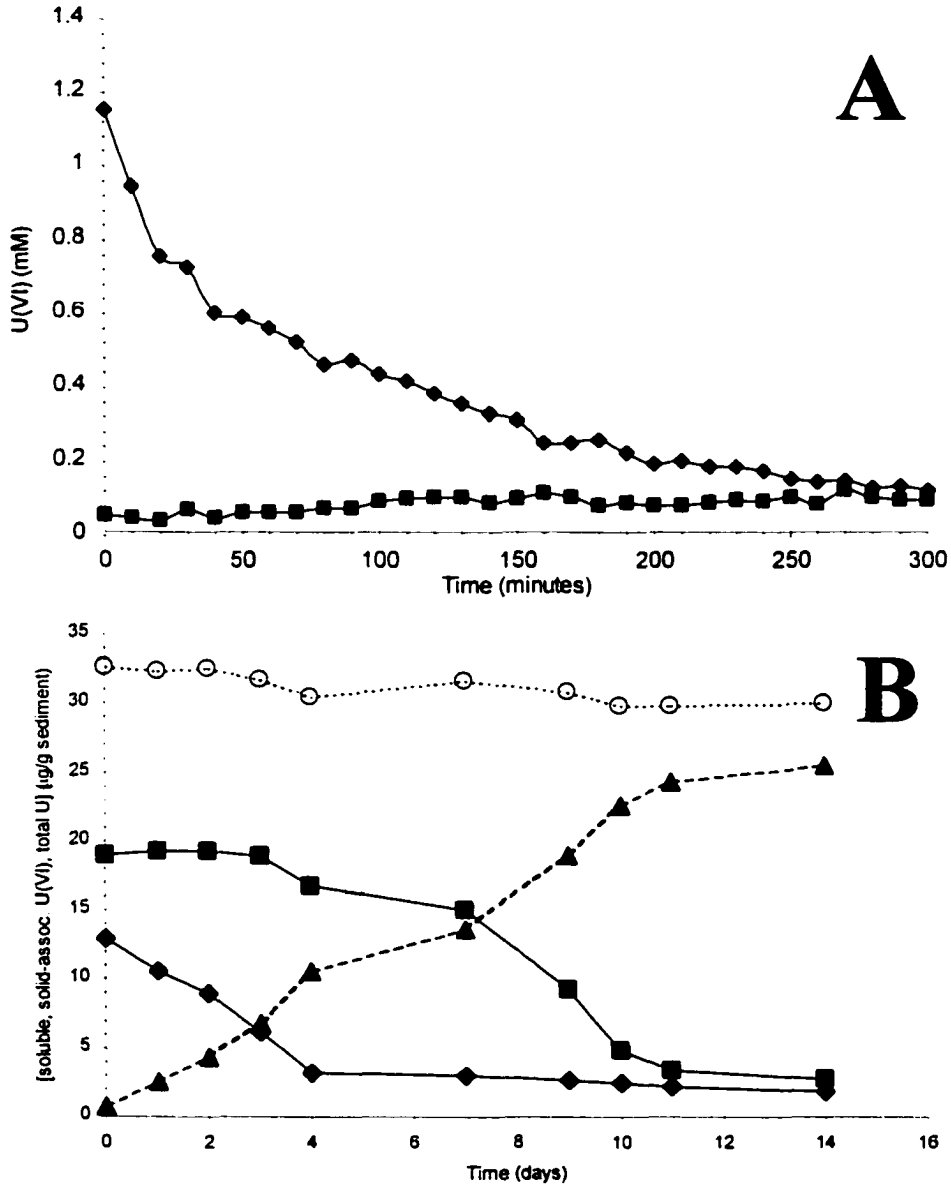


Figure 5: U(VI) concentrations in soluble (◆) and complexed (■) fractions during a U(V)-reduction assay with (A) *D. desulfuricans* Essex6. (B) U-reduction in subsurface sediments with total uranium levels (O), complexed U(VI) levels (■), soluble U(VI) (◆), and HNO_3 extractable U (assumed to be U(IV)) (▲).

Chapter 3

The periplasmic cytochrome c_3 of *Desulfovibrio vulgaris* is directly involved in H_2-mediated metal-, but not sulfate-, reduction

Abstract

Kinetic parameters for sulfate- , Fe(III)- , and U(VI)- reduction as well as the role of cytochrome c_3 in these processes was investigated in *Desulfovibrio vulgaris* Hildenborough. While sulfate-reduction followed Michealis-Menten kinetics (K_m=220 μM), loss of Fe(III) and U(VI) was first-order. The specific activity for all three electron acceptors was similar when cells were grown on H_2/sulfate, while cultures grown using lactate/sulfate had similar levels of Fe(III) and U(VI) loss, but lower sulfate-reduction activity. The similarity in metal- , but not sulfate- , reduction activity with H_2 and lactate suggests a divergence of pathways. Specific activities decreased substantially when cells were grown fermentatively. Metabolic assays and reduced minus oxidized cytochrome spectra were carried out to determine the role(s) of cytochrome c_3 in electron acceptor reduction. Cytochrome oxidation was immediate with Fe(III) and U(VI) in the presence of H_2, lactate, or pyruvate as electron donors. Sulfidogenesis occurred

with all three electron donors, and effectively oxidized the cytochrome in lactate or pyruvate, but not H_2 reduced cells. Correspondingly, electron acceptor competition assays with lactate or pyruvate as electron donors showed that Fe(III) inhibited U-reduction, and U(VI) inhibited sulfate loss. However, the reduction of sulfate was slowed but not halted when H_2 was the electron donor in the presence of Fe(III) or U(VI). Uranium loss was still impeded by Fe(III) when H_2 was used. Hence we present a modified pathway for the reduction of sulfate, Fe(III), and U(VI) which helps explain why these bacteria cannot grow using these metals. We propose that cytochrome c_3 is an electron carrier involved in lactate/pyruvate oxidation and is the reductase for alternate electron acceptors with higher redox potentials than sulfate. As such, the role of the cytochrome may be to protect the cell against oxidation.

Introduction

The electron transfer pathways and participation of the periplasmic cytochrome c_3 in sulfate-reduction have been intensively investigated (3, 5, 12, 15-17, 19, 20, 24, 35, 38, 40, 42, 59, 62). Sulfate-reducing bacteria oxidize lactate or pyruvate to acetate, hydrogen, and CO_2 (45, 46, 71). A H_2 cycling model was proposed (41) to explain H_2 production, but could only account for 48% of the electrons transported from lactate (39). Subsequently, a unified model described lactate oxidation to acetate by combining three pathways, one involving H_2 cycling and two using electron carriers (39).

The most intensely studied of these carriers in *Desulfovibrio (Dsv.) vulgaris* are cytochromes (61, 67), with the foremost being the periplasmic cytochrome c_3 (38, 51). The latter is used as a biomarker for *Dsv.* spp. (38). Similarities that exist between strains include heme 4 interacting with the hydrogenase for electron transfer (2, 5, 21, 48), as well as an N-terminal amino acid sequence extension for periplasm export (2, 21, 26, 68, 69).

Recently a periplasmic facing, but membrane associated cytochrome c_3 was found in *Dsv. vulgaris* Hildenborough (66) with similar E^o values and reduced minus oxidized spectrum to the soluble protein (7). Rather than being regarded as a general electron carrier, the more recently described cytochrome is speculated to function with the high molecular weight complex (*hmc*) as a membrane bound oxidoreductase complex since they are part of the same operon. The periplasmic cytochrome c_3 on the other hand functions as a reductase for alternate electron

acceptors such as O_2 (11, 12, 25, 35), Cr(VI) (32), Fe(III) (30), Zn(II) (18), and U(VI) (34).

We are specifically interested in how sulfate-reducing bacteria reduce U(VI) to U(IV). While these microorganisms can tolerate up to 24 mM uranium (30), they cannot grow using it as the sole electron acceptor (64). Exceptions are *Dsv. vulgaris* UFZ B 490 (49), and *Desulfotomaculum reducens* (63). Reports of the kinetic parameters for U-reduction showed enhanced rates in the presence of sulfate by a sulfate-reducing enrichment (specific activity of 23 $\mu M\ min^{-1}$ mg $cells^{-1}$, apparent K_m of 250 μM) (57, 58), while *Dsv. desulfuricans* showed no such stimulation (specific activity of 30 $\mu M\ min^{-1}$ mg $cells^{-1}$, K_m of 500 μM) (31, 57). In both cases, sulfate and uranium were reduced concomitantly. Whereas *Dsv. desulfuricans* showed zero-order kinetics for sulfide production with or without U(VI), loss of the latter was first-order (58) indicating that U-reduction is not a typical enzymatic reaction.

Given the similarity between strains, the ability to reduce numerous potential electron acceptors, along with first-order U-reduction kinetics, the periplasmic cytochrome c_3 may be more than a simple electron carrier. Here we report for the first time that this cytochrome is likely involved in sulfate-reduction from lactate and pyruvate, but not H_2 in *Dsv. vulgaris* Hildenborough. Further, Fe(III) and U(VI) completely inhibit lactate and pyruvate mediated sulfate-reduction, but only slow this process when H_2 is the electron donor. By using washed cells in reduction assays and cytochrome spectra, the evidence indicates at least two pathways for sulfidogenesis with a specific role for cytochrome c_3. Finally, we

present a modified electron transport pathway where both the present work and previous reports agree.

Methods

Growth and harvesting of bacteria

Cultures of *Dsv. vulgaris* Hildenborough (ATCC 29579), as well as the wild type and cytochrome c_3-deficient mutant of *Dsv. desulfuricans* G20 (52) (a gift from J. Wall) were grown with the medium previously described (43). The latter was grown in the presence of 1mg/ml kanamycin to maintain the cytochrome c_3 mutant. Cultures were grown with either 20 mM Na-lactate or H_2 as the electron donor and sulfate as the electron acceptor, or fermentatively with 20 mM Na-pyruvate. Once grown ($A_{660} \simeq 0.80$), cultures were anaerobically harvested and centrifuged (27,000 x g, 20 min, 10°C). Cells used for experiments with lactate or pyruvate as the electron donor were washed and centrifuged 3 times in 30 mM $NaHCO_3$ buffer, pH 6.8 (34). When H_2 was the electron donor, cysteine ($0.25gL^{-1}$) was added to the buffer to facilitate H_2/sulfate-reduction (31). After washing, the pellet was resuspended in 15 ml of the appropriate buffer per liter of culture.

Use of HCl to preserve electron acceptor concentrations

In order to accurately follow electron acceptor reduction over time, different strengths of HCl (under N_2) were used to determine the appropriate concentration to stop the reaction without interfering with U(VI) quantitation. Uranyl-carbonate (100 μM) was prepared (33) and 0.2 ml of U(VI) solution was added to 0.2 ml of each HCl concentration (1.2 – 12M). Samples were quantitated for U(VI) using a

kinetic phosphorescence analyzer (Model KPA-11, Chemchek Instruments, Inc.) as described previously (4, 37).

Modeling of U(VI) and Fe(III) concentrations

The major species of U(VI) and Fe(III) in our experiments were determined using Phreeqci (http://water.usgs.gov/software/phreeqci.html) and the Lawrence Livermore National Laboratory database. The model was also used to argue that the electron acceptors remained in solution and were likely bioavailable. Concentrations of 2 mM were used to model reduction experiments.

Kinetics of Fe(III), sulfate, and U(VI) reduction

The kinetic of sulfate- , Fe(III)- , and U(VI)- reduction were compared to first-order, and Michealis-Menten models. The slope of the semi-logarithmic decay curves was used to determine the first-order decay coefficients. The first-order reactions were determined in less than 1 h. The apparent K_m and V_{max} associated with sulfate-reduction was determined using the integrated Michealis-Menten equation (55).

Variation of U(VI) concentrations

In order to calculate the specific activity of U-reduction and to determine if saturation could be achieved, the concentrations of U(VI) was varied (0.05 – 5 mM) in a reaction mixture containing 8 ml of cell concentrate. The cells were added to 92 ml of 2 mM uranyl-carbonate in 30 mM $NaHCO_3$ buffer, pH 6.8 with

20 mM lactate as electron donor (the assay solution). Samples were taken as above every 20 min for the first 6 h and periodically thereafter.

Electron acceptor reduction assays

Assays were carried out with the same electron donor as was used to grow the cells. The loss of sulfate, Fe(III), and U(VI) in cell concentrates was followed over time by periodically removing 0.2 ml of cell suspension, and injecting into 0.2 ml of anaerobic 1.2 M HCl. Samples were centrifuged (13,000 x g; 10 min; 20°C) and the supernatant removed for quantitation. The loss of U(VI) was monitored as described above, while sulfate concentrations were measured using a Dionex DX-500 ion chromatograph with an AS4A anion column as previously described (65). Levels of Fe(II)/Fe(III) were determined by a colorimetric method (29). Assays with *Dsv. vulgaris* used lactate, pyruvate, or H_2 as the electron donor, while those with *Dsv. desulfuricans* G20 involved H_2. Lactate was measured using a Dionex DX-500 chromatography system with an AS-11A anion column (56). In order to overcome H_2 phase-transfer limitations and ensure electron acceptors remained in solution, stir bars were placed in serum bottles (160 ml) prior to flushing (N_2/CO_2) and autoclaving, and the reaction mixture constantly stirred. Once cool, filter-sterilized anaerobic solutions of U(VI) (as uranyl-carbonate), $FeCl_3$, or Na_2SO_4 were added in the appropriate concentration and volume (see below). Assay solutions were made in 30 mM $NaHCO_3$ (+/- 0.25g/L cysteine) buffer, pH 6.8, with a total volume of 100 ml. All manipulations

were conducted in a well working anaerobic chamber except for electron acceptor measurement. Experiments were conducted in duplicate.

Competition for electron acceptors

Competition assays were performed to see if *Dsv. vulgaris* could co-reduce U(VI) or Fe(III) along with sulfate. If sulfate loss was blocked while metal-reduction occurred, this would argue for electrons being utilized from a common electron transfer component. Assays were carried out (as described above) with 20 mM lactate (N_2/CO_2) or H_2 (H_2/CO_2) as electron donor, with 2 mM Fe(III) and U(VI), Fe(III) and sulfate, or U(VI) and sulfate. Positive controls used Fe(III), U(VI), or sulfate alone along with heat-killed negative controls. Time course aliquots were sampled as above.

Inhibition of sulfate-reduction vs. U(VI)/Fe(III)-reduction

Molybdate has often been shown to inhibit sulfate-reduction, with the proposed site of inhibition being the APS-reductase (44). In order to determine if U(VI)- and/or Fe(III)- reduction occurred prior to electrons reaching the APS-reductase, 20 mM Na-molybdate was added to reduction assays with 2 or 20 mM Na_2SO_4, 2 mM uranyl-carbonate, or 2 mM $FeCl_3$. Assays were performed as above with lactate as the electron donor.

Effect of periplasm removal on electron acceptor reduction

The proposed U(VI)-reductase, and most likely the Fe(III)-reductase (34), is the periplasmic cytochrome c_3.The periplasm of lactate/sulfate grown *Dsv. vulgaris* cells were removed (3) to show that these components were required for these activities as well as sulfate-reduction. The cells were resuspended in 15 ml/L culture of 30 mM $NaHCO_3$, pH 6.8. Cells were inspected microscopically to ensure that cell lysis had not taken place.

Lactate dehydrogenase activity of treated cells was tested as above with lactate as the electron donor and methylviologen as electron acceptor. Lactate was measured as above. Methylviologen reduction was measured spectroscopically at 560 nm (54, 70) to record an increase in reduced methylviologen.

Cytochrome c_3 spectra

The oxidation of the periplasmic cytochrome c_3 via sulfate, U(VI), or Fe(III) was determined for both *Dsv. vulgaris* and the cytochrome c_3-deficient *Dsv. desulfuricans* G20 mutant using a modified reduced minus oxidized spectral method (36). Sample cuvettes were constructed using 12 x 75 mm glass test tubes sealed with 00 rubber stoppers, flushed with N_2/CO_2 or H_2/CO_2, and autoclaved. A spectrophotometer (Beckman DU64; Beckman Coulter, Inc.) scanned the assay mixture from 400 – 650 nm, at 500 nm/min. The reduced baseline was obtained by adding 0.2 ml of the cell concentrate to 1.7 ml of sterile 30 mM $NaHCO_3$ buffer (+/- cysteine), with a reductant. The reducing agent was Na-dithionite, 40 μM lactate or pyruvate under N_2/CO_2, while a headspace of H_2/CO_2 was used for

experiments involving H_2. The cells were allowed to incubate in the dark (1 h). Prior to electron acceptor addition, the H_2/CO_2 gas phase was aseptically exchanged to N_2/CO_2 to limit electron generation and facilitate cytochrome oxidation. After incubation, 0.1 ml of 25 mM Na_2SO_4, uranyl-carbonate, or $FeCl_3$ (2 mM final concentration) was added, and the spectrum recorded immediately and periodically thereafter for 2 h.

Coordinating sulfidogenesis with cytochrome c_3 oxidation spectra

The oxidation of periplasmic cytochrome c_3 via sulfate-reduction from lactate, pyruvate, or H_2 (+/- cysteine) was matched, with respect to time, to the production of sulfide. This was done to determine if sulfidogenesis occurred without cytochrome oxidation. The spectral assays were performed as described above, however, immediately prior to scanning the oxidized spectrum, 0.1 ml of cuvette contents were removed via syringe and added to 0.5 ml of 4% Zn-acetate to quantitate sulfide (8).

Results

Use of HCl to preserve electron acceptor concentrations

Quantitation of U(VI) with rising HCl concentrations showed that the KPA response was increasingly quenched (data not shown). Little signal loss occurred with 6 mM (final concentration), so 1.2 M HCl was used 1:1 (v:v) with subsequent 1:100 fold dilution in water prior to analysis to preserve electron acceptor levels at each time point.

Modeling of U(VI) and Fe(III) concentrations

Phreeqci modeling for the addition of 2 mM uranyl-carbonate or $FeCl_3$ to 30 mM $NaHCO_3$, pH 6.8 resulted in two major species for each electron acceptor. In the case of uranyl-carbonate, $UO_2(CO_3)_3^{4-}$ (1.079 mM) and $UO_2(CO_3)_2^{2-}$ (0.787 mM) made up 93.3% of the U(VI) added. For Fe(III), $Fe(OH)_3$ (1.448 mM, likely as a mineral based on the saturation index) and $Fe(OH)_2^+$ (0.550 mM) made up greater than 99% of the Fe(III) present. This indicated that rather than several species being involved, two were dominant and except for the $Fe(OH)_3$ were deemed bioavailable based on preliminary reduction assays.

Kinetics of Fe(III), sulfate, and U(VI) reduction

Progress curves for sulfate consumption followed apparent Michealis-Menten kinetics (Figure 1A). This is shown most obviously in the insert where the semilogarithmic plot of the same data shows clear zero- and first- order decay

regions. Analysis of data reveals an apparent K_m and V_{max} of 0.22 mM and 0.35 mM h^{-1}, respectively. Substantially different kinetics were observed when the same culture was incubated with Fe(III) or U(VI). With starting concentrations of Fe(III) as high as 1 mM, no hint of saturation was observed (Fig. 1B). A semilogrithmic plot reveals that Fe(III) reduction is a first-order process with a proportionality constant ranging from 0.89 - 0.91 h^{-1} for 1 mM Fe(III). Similarly, when U(VI) reduction was assayed, first-order kinetics was also observed at concentrations ranging from 0.05 -0.3 mM (Fig. 1C). At these concentrations, the first-order decay rate ranged from 2.7 – 4.2 h^{-1}, while 2 mM U(VI) showed a rate of 1.65 h^{-1} (insert). When higher concentrations were used, no saturation was observed up to 5 mM U(VI). Rather, at the latter concentration uranium reduction was inhibited to the point where a measurable rate could not be detected (Figure 1C).

Electron acceptor reduction assays

The loss of U(VI), Fe(III), and sulfate were monitored over time in washed cell suspensions. Cells grown with H_2/sulfate and assayed with H_2 as electron donor showed similar initial rates for sulfate-, Fe(III)- , and U(VI)- reduction. However, several times more electrons are required for sulfate- than for metal-reduction (Table 1), suggesting their preferential flow to sulfate. The rate of Fe(III) and U(VI) loss was unchanged with lactate as a donor. Under the latter conditions, the electrons used for sulfate-reduction were about half of that used when H_2 served as a donor, indicating a reduction process that was separate from

metal reduction. The activities using pyruvate were lower for Fe(III)- and U(VI)-reduction, with no appreciable difference in sulfate-reduction compared to lactate. When the *Dsv. desulfuricans* G20 cytochrome c_3-deficient mutant was grown on lactate/SO_4, lactate or pyruvate-dependent U(VI)-reduction rates were slower than the wild type, while there was no difference in H_2-dependent U(VI)-reduction rates between the wild type and mutant when grown on H_2/SO_4 (data not shown).

Competition for electron acceptors

The periplasmic cytochrome c_3 reportedly functions both as the U(VI)-reductase (34) and as an electron carrier for sulfate-reduction (5, 60). Assays were performed to determine if the presence of one electron acceptor would impact the reduction of another. The rates and activities for control assays of Fe(III), U(VI), and sulfate alone were similar to previous experiments, while no activity was observed in heat-killed controls (data not shown). When lactate was the electron donor, sulfate-reduction was inhibited in the presence of U(VI) until the rate of loss of the latter began to decrease (Fig. 2A). Similarly, U(VI)-reduction was halted by the presence of Fe(III) (Fig. 2B). However with H_2 as electron donor, U(VI) only slowed sulfate-reduction (59.7% inhibition) until U(VI) loss ceased (Fig. 2C). The presence of Fe(III) inhibited U(VI)-reduction (Fig. 2D) by H_2 as was observed with lactate as donor.

Inhibition of sulfate-reduction vs. U(VI)/Fe(III)-reduction

The addition of 20mM Na-molybdate inhibited lactate-mediated sulfate-reduction with both 2mM (Fig. 3) and 20mM (data not shown) Na_2SO_4 as expected. However, the presence of molybdate did not influence Fe(III)- or U(VI)-reduction (Fig. 3). This indicated that electron transfer to Fe(III) and U(VI) does not involve components that are inhibited by molybdate, and supports a separation of the sulfidogenic pathway from that of metal-reduction.

Effect of periplasm removal on electron acceptor reduction

In order to confirm that contents of the periplasm are essential for electron transport to reduce Fe(III), U(VI), and sulfate, this part of the cell was removed and the periplasmless cells tested against intact ones. In each case, the intact cells reduced all 3 electron acceptors at rates similar to previous assays while periplasm removal stopped all activity (data not shown). The cytoplasm of the treated cells was still intact as lactate was oxidized with the concomitant reduction of methylviologen at rates similar to intact cells (data not shown).

Cytochrome c_3 spectra

The cytochrome c_3 spectrum for *Dsv. vulgaris* showed the expected Soret peak at 419 nm, with α- and β- peaks at 553 nm and 525 nm, respectively. Reduction of the cytochrome using dithionite, H_2/CO_2, lactate, and pyruvate was successful after incubation (1 h) since reduced minus oxidized spectra were seen

when the cuvettes were exposed to air. The addition of 2 mM Fe(III) , U(VI), or sulfate caused immediate oxidation of the cytochrome when reduced with lactate, pyruvate, or dithionite confirming that involvement of this protein in electron transfer from organic donors. The coupling of H_2 with the electron acceptors was not as straightforward. Addition of 2 mM Fe(III) or U(VI) to H_2 reduced cells showed an immediate cytochrome oxidation spectrum, while none was seen with sulfate addition after a 2 h incubation (data not shown).

When sulfide production assays were coupled to these spectra, cells grown with either H_2/ or lactate/ sulfate generated similar sulfide concentrations, indicating the method of growth was not a factor in these experiments. Sulfidogenesis by washed cells was immediate and similar using lactate or pyruvate as electron donors, and was concomitant with cytochrome oxidation spectra (Fig. 4A,B). However, while 50 μM sulfide was produced with H_2 reduced cells, no differential spectrum was observed after 2 h (Fig. 4C), showing that cytochrome c_3 is not involved in H_2-mediated sulfate-reduction.

Discussion

Several *Desulfovibrio* spp. are capable of reducing both Fe(III) (30) and U(VI) (57), but are unable to grow using these electron acceptors (64). Previous work reported that Fe(III)-reduction occurs via the periplasmic cytochrome c_3 (30), and that both a soluble hydrogenase and this cytochrome were required for *in-vitro* U(VI)-reduction (34). Here we report that U(VI)-reduction (up to 5 mM) was possible, but rates dropped sharply as concentrations fell below 300 μM as previously observed (57). By using various models, the data agrees with previous results suggesting that both U(VI)- (57, 58) and Fe(III)- (27) reduction are first-order processes, although a half-saturation constant of 29 mM was calculated for *Shewanella putrefaciens* CN32 (28). The process order is important with regard to using sulfate-reducing bacteria for uranium bioremediation. Since the cells cannot derive energy from U(VI)-reduction (64) and the substrate concentration alone will determine the reaction rate, a reaction carried out by these microorganisms will be highly predictable (55).

A comparison of the Soret, α, and β peaks from our reduced minus oxidized cytochrome spectra with previous data (51) confirmed measurement of cytochrome c_3 oxidation, and not the *hmc* (6), cytochrome c_{553} (67), or cytochrome c_7 (61), providing further evidence that cytochrome c_3 is the U(VI)- (34) and Fe(III)- reductase (30). This cytochrome has also been implicated in O_2-reduction in sulfate-reducing bacteria (11, 12), where sulfidogenesis was interrupted until O_2 exhaustion (35). Our results provide similar conclusions regarding the exhaustion of Fe(III) and U(VI) before sulfate-reduction will occur.

Thus, it could be suggested that the periplasmic cytochrome c_3 functions in a protective role (9) whereby alternate electron acceptors with higher redox potentials than that of sulfate (-220mV), are immediately reduced to prevent cell oxidation. This could imply that electron transfer to the cytochrome is a 'dead-end pathway', but since it plays an essential role in pyruvate/sulfate-reduction (52), function as a central electron carrier (13, 40) is more likely. This point is illustrated as pyruvate or lactate reduced cytochrome c_3 is oxidized by Fe(III), U(VI), and sulfate to suggest a common pathway for metal- and sulfate- reduction from organic electron donors. However, use of H_2 revealed a separation of pathways where Fe(III) still inhibited U(VI)-reduction, but sulfate loss was slowed instead of being halted by either of the metals. A reduced minus oxidized cytochrome spectrum for H_2/Fe(III)- or U(VI)- reduction, but not for that of sulfate, suggests that while some electrons are donated from H_2 to reduce cytochrome c_3, the majority are involved in H_2-dependent sulfate-reduction and do not necessarily involve the cytochrome (14, 23, 50, 53, 59).

Hence, given the reports cited above along with the present results, we propose the pathway shown in Figure 5. In this scheme, exogenous H_2 is oxidized by both the Fe-only and NiFe hydrogenases. Electrons from the latter are donated to periplasmic cytochrome c_3 (13, 40) for O_2- , Fe(III)- , or U(VI)- reduction, while those from the Fe-only hydrogenase are donated to *hmc* without cytochrome c_3 participation. This lack of involvement is supported by *hmc* deletion inhibiting H_2/, but not lactate or pyruvate/sulfate-reduction (14), as does

the fact that H_2-dependent U(VI)-reduction, but not growth was drastically affected by cytochrome loss (47).

Given the poor growth of the cytochrome c_3 mutant with pyruvate, but not lactate or H_2 (52), its' reduced U(VI)-reduction ability with pyruvate and H_2 (47), and our competition and spectra experiments, electrons from pyruvate are likely funneled through cytochrome c_3. The mutants' ability to reduce U(VI) in a diminished capacity and the presence of the spectrum can be explained if multiple isozymes of the cytochrome are present. In our model, pyruvate oxidation indirectly produces H_2, and this may occur via electron transfer through cytochrome c_3 to the NiFe hydrogenase, which is reversible in other bacteria (22).

Electron flow from lactate oxidation also results in H_2 production (50), but does not always involve cytochrome c_3 since its' deletion had no effect on lactate/sulfate-reduction (52). However, Fe(III) and U(VI) inhibited lactate/sulfate-reduction in our assays, and cytochrome c_3 reduced with lactate was oxidized with Fe(III), U(VI), and sulfate. Hence, we propose that electron flow from the membrane-bound lactate dehydrogenase (LDH) (10) is not tightly coupled to cytochrome c_3, but that electrons are also shuttled from LDH to the ech-hydrogenase for H_2 generation (50).

Finally, our model helps explain why these microorganisms cannot grow using Fe(III) or U(VI) as the terminal electron acceptor. Analysis modeling of the chemical species involved suggested that $Fe(OH)_2^+$, $UO_2(CO_3)_3^{4-}$, and $UO_2(CO_3)_2^{2-}$ are the major soluble components in the reaction mixtures. Since these metals are reduced in the periplasm (34), the reduction of each will result in

a diminished proton gradient. During Fe(III)-reduction, a hydroxyl group will be released and combine with available protons. Similarly, since uranyl-carbonate is the favored U(VI) form at neutral pH (1), UO_2^{2+} reduction ($UO_2(CO_3)_3^{4-}$) to UO_2 will free negatively charged carbonate which will also take up protons. Thus, it would be much more difficult to maintain a proton gradient during metal-respiration, but it is a necessary expenditure to prevent oxidation inside the cell.

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Table 1: Comparison of initial rates of electron acceptor reduction with growth conditions for *Dsv.vulgaris* (Hildenborough)

	Growth Condition / Electron donor and acceptor		
	H_2/sulfate	lactate/sulfate	pyruvate fermentation
Electron acceptor	Initial Rate ($\mu M\ min^{-1}\ mg\ protein^{-1}$)	Initial Rate ($\mu M\ min^{-1}\ mg\ protein^{-1}$)	Initial Rate ($\mu M\ min^{-1}\ mg\ protein^{-1}$)
Fe(III)	12.2 (12)*	12.3 (12)	7.7 (8)
U(VI)	16.0 (32)	15.9 (32)	4.8 (10)
Sulfate	11.8 (96)	7.3 (58)	7.1 (56)

*- values in parentheses indicate the equivalent number of electrons transferred over the time course of measurement per mg protein.

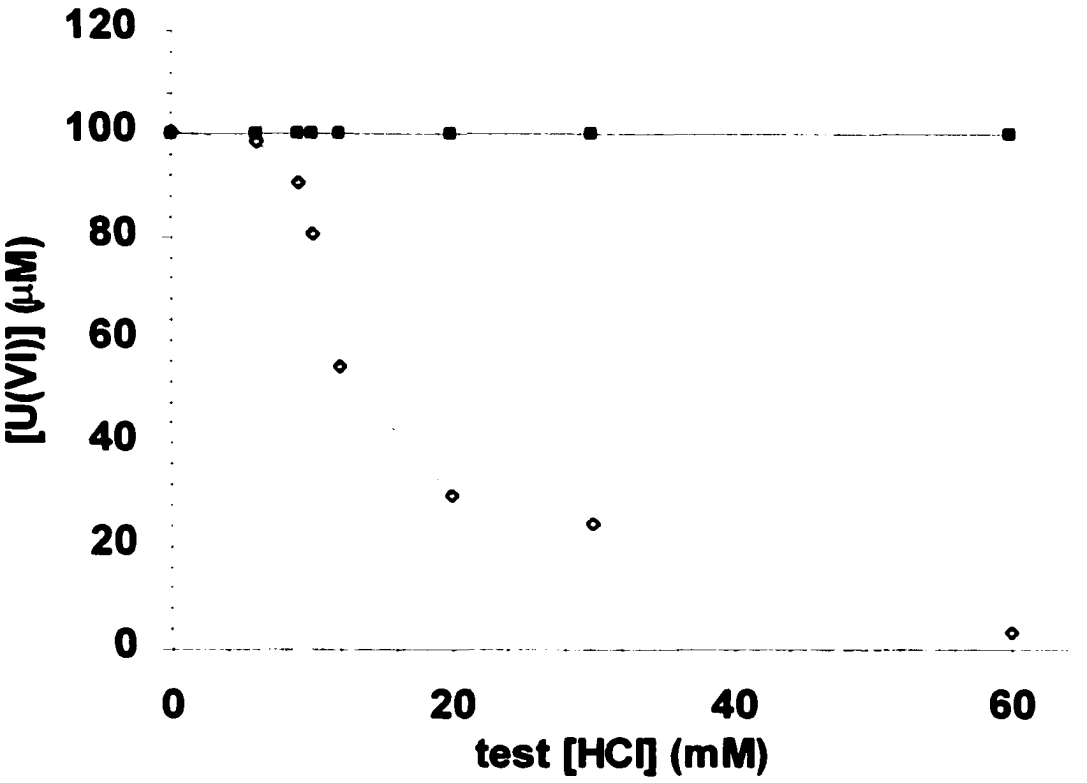

Figure 1: The effect of HCl quenching on U(VI) quantitation in tested (◊) vs. theoretical (■) values.

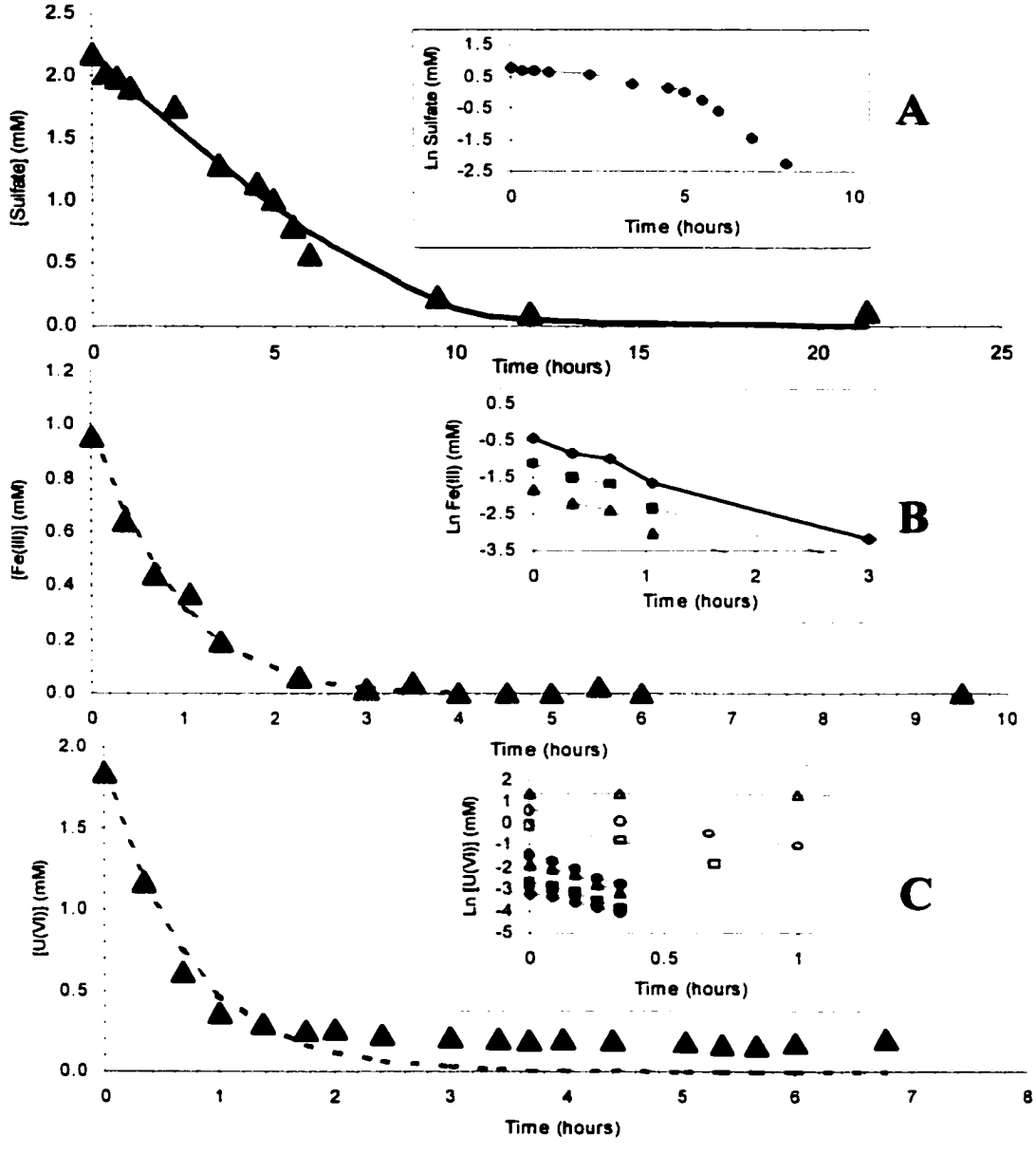

Figure 1: A comparison of the kinetics of (A) sulfate, (B) Fe(III), and (C) U(VI) reduction by washed cells. The inserts are semilogarithmic plots of the respective reduction processes for sulfate (2 mM), U(VI) (0.05 – 5.0 mM), and Fe(III) (0.2 – 1.0 mM). The solid line in frame A is a model of the sulfate depletion curve based on the Michealis-Menten equation, the derived K_m and V_{max} values, and a initial starting concentration of 2 mM. First-order decay curves are modeled (broken lines) in B and C based on the measured decay coefficient and the starting concentration.

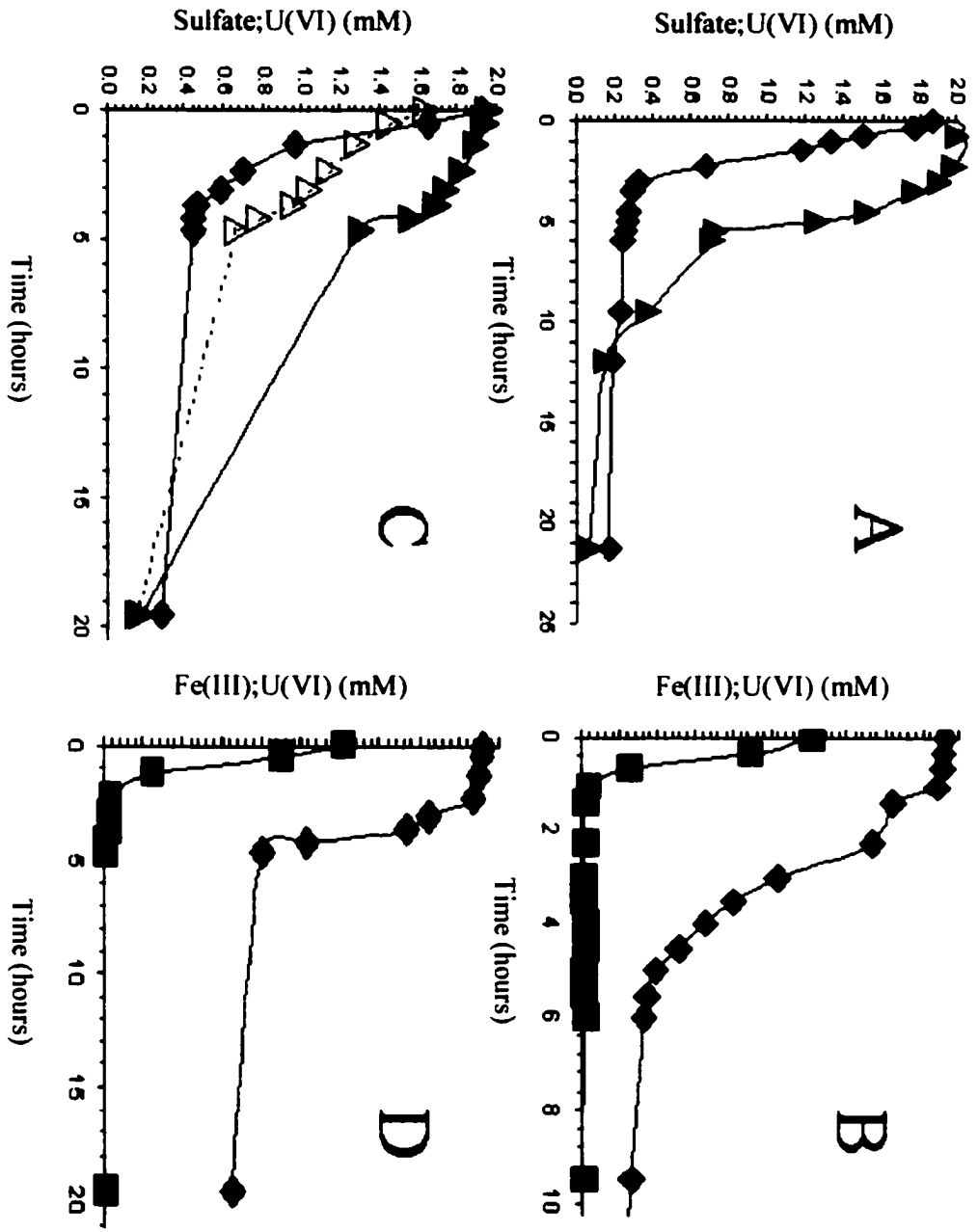

Figure 3: The inhibition of the reduction of (A) sulfate (▲) by U(VI) (◆), and (B) U(VI) (◆) by Fe(III) (■) when lactate was the electron-donor. When H_2 was used as the electron-donor, the reduction of (C) sulfate (▲) in the presence of U(VI) (◆) was slowed compared to sulfate alone (Δ). The reduction of (D) U(VI) (◆) was still impaired by Fe(III) (■).

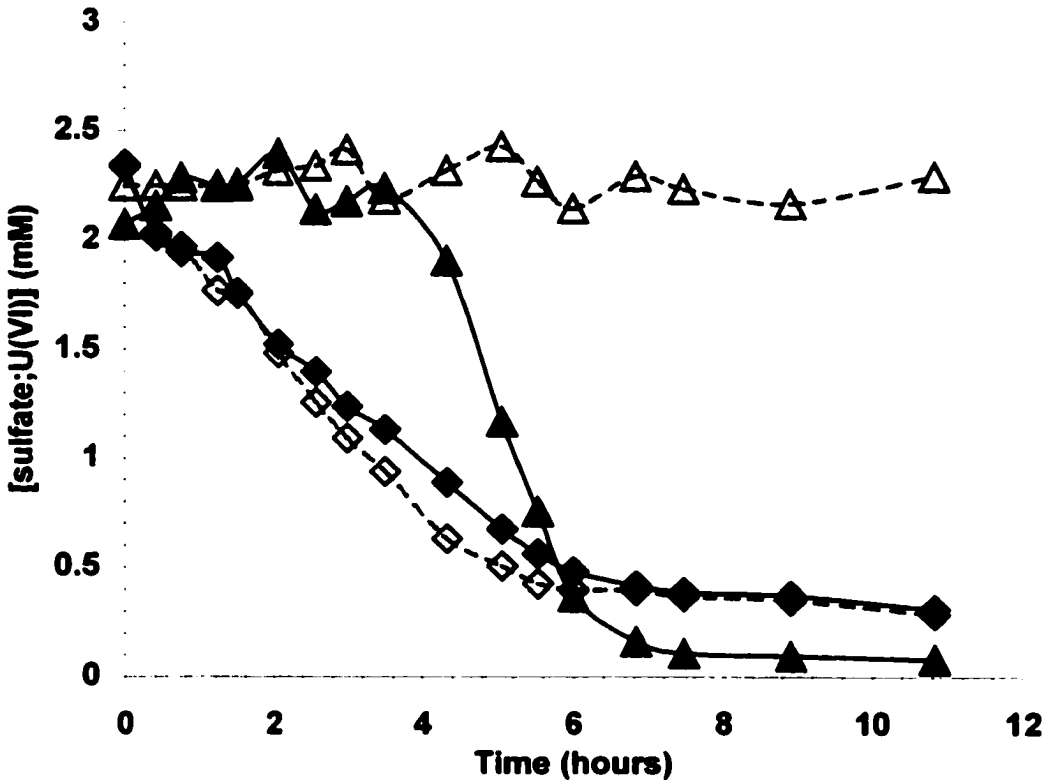

Figure 4: The presence of molybdate completely inhibited the reduction of sulfate (Δ) as opposed to the transient inhibition of U(VI) on sulfate-reduction (▲). The presence of molybdate + U(VI) + sulfate (◊) had no effect on U(VI)-reduction when compared to U(VI) + sulfate (◆).

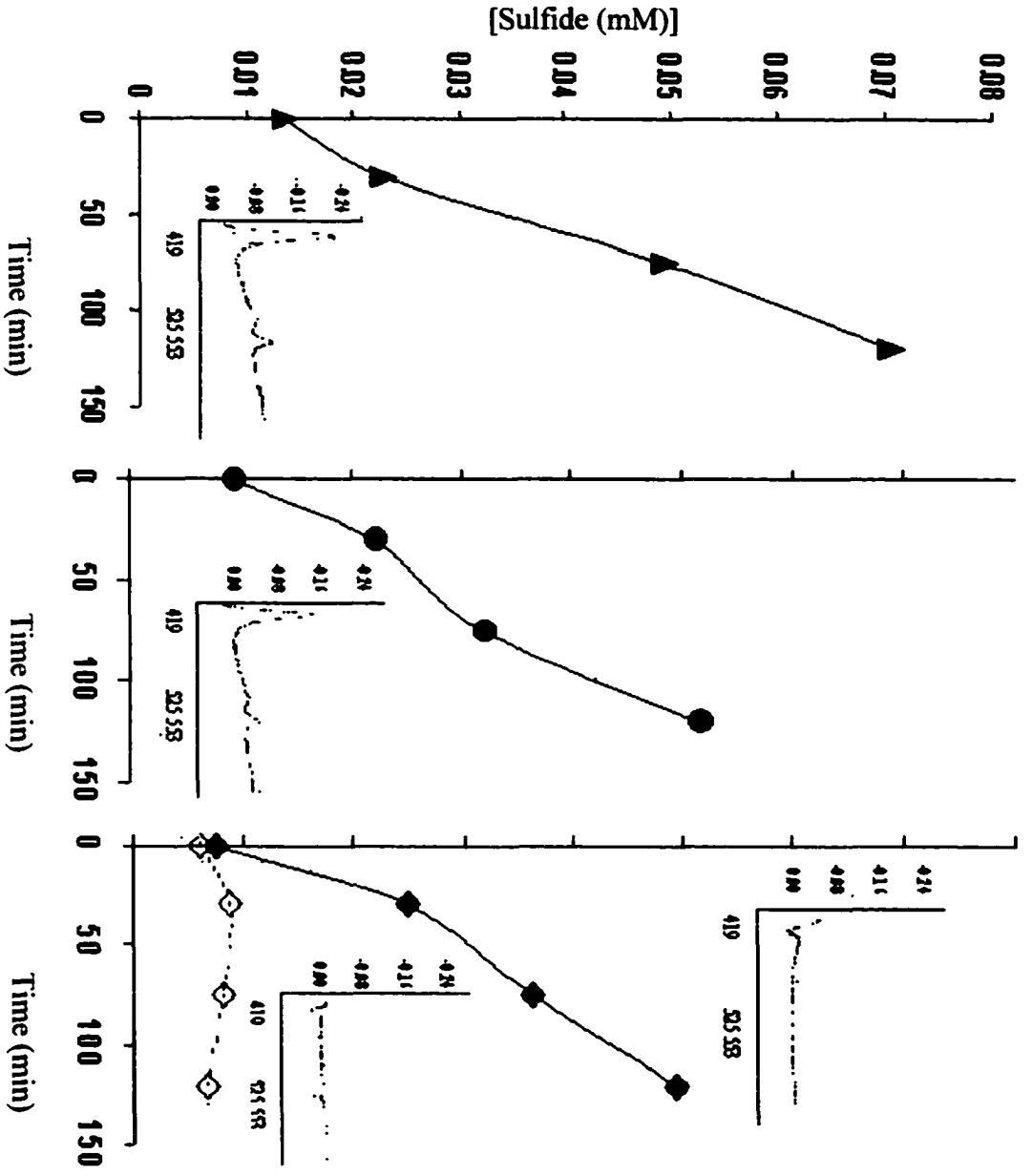

Figure 5: The production of sulfide monitored over time with concomitant reduced – oxidized cytochrome spectra. Sulfidogenesis was found when lactate (▲), pyruvate (●), and H_2 + cysteine (◆), but not H_2 - cysteine (◊) were used to reduce the cytochrome. Inserts show the spectrum obtained after 30 min from lactate and pyruvate, and after 2h from H_2 +/- cysteine.

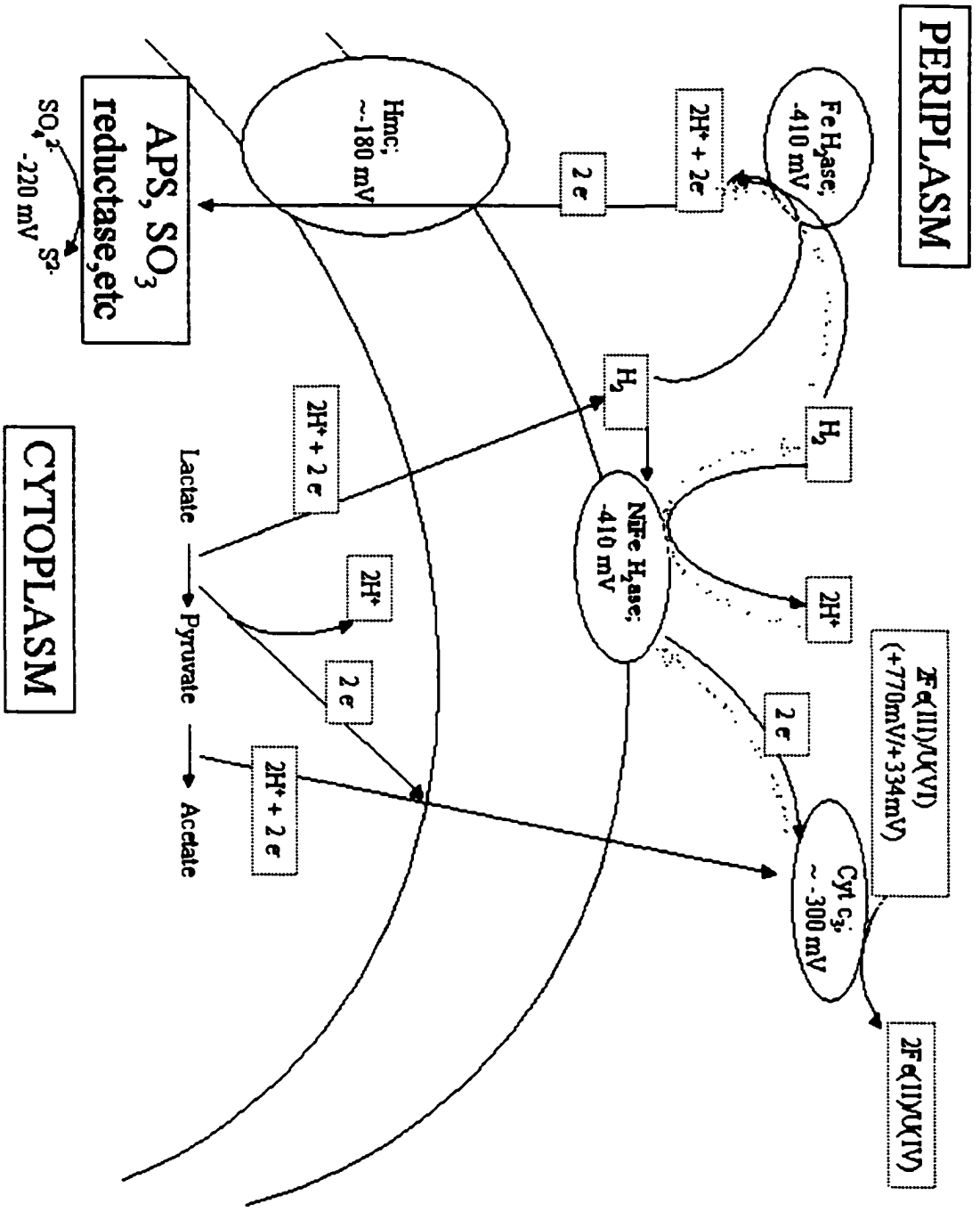

Figure 6: Electron transfer for sulfate-reduction from H_2, vs. lactate and pyruvate. Greyed lines indicate the formation of H_2 from lactate or pyruvate via cytochrome c_3 and NiFe hydrogenase. Fd=ferrodoxin, ECP=electron-carrier protein, H_2ase=hydrogenase.

Appendix A

Estimation of Methanogen Biomass by Quantitation of Coenzyme M

Abstract

A determination of the role of methanogenic bacteria in an anaerobic ecosystem often requires their quantitation. Because of the extreme oxygen sensitivity of these organisms and the inherent limitations of cultural techniques, an accurate biomass determination is very difficult to obtain. We have standardized a simple method for the estimation of methanogen biomass within a variety of environmental matrices. This procedure uses the thiol biomarker, Co-enzyme M (CoM; 2-mercaptoethanesulfonic acid), which is known to be present in all methanogenic bacteria. An HPLC based method for the detection of thiols in pore water (30)has been modified to quantify CoM in pure cultures, sediments and sewage water samples. The identity of the CoM derivative was verified using Liquid Chromatography/Mass Spectroscopy. The assay is linear from 2-2000

pmoles CoM with a detection limit of 2pmoles CoM/ml sample. CoM was not adsorbed to sediments. Methanogens tested averaged 19.5nmol CoM/mg protein, and 0.39±0.07 fmol CoM/cell. Environmental samples averaged 0.41±0.17 fmol/cell based on MPN estimations. Extraction of CoM was achieved using 1% tri-(N)-butylphosphine in isopropanol. Recovery of greater than 90% was achieved with pure cultures and environmental samples. We encountered no interference from sediments in CoM recovery and the method can be completed aerobically within 3h. Freezing of sediment samples resulted in 46-83% decrease of the detectable CoM, whereas freezing had no effect on CoM determinations in pure cultures. This method provides a quick and relatively simple means of estimating methanogenic biomass.

Introduction

Methanogenesis occurs in a wide variety of anaerobic habitats including wetlands, lake sediments, animal gastrointestinal tracts, and geothermally heated environments. Optimal temperatures for methanogenesis vary from 2°C to over 100°C. Most methanogens can use H_2 or acetate as a source of electrons for methanogenesis. In this respect, these organisms carry out the terminal step in anaerobic decomposition (34).

Methanogens thus play an essential role in the mineralization of organic material (5,10). They are directly involved in the treatment of wastewater, sewage, and solid wastes (14,18,19,23-25).

Ecological and environmental studies often demand a quick, easy, and reliable method for accurately estimating methanogenic biomass. Methanogens possess several potential biomarkers including F_{420}, isoprenoids and lipid ethers in their cell membranes, and coenzyme M (CoM). While F_{420} has been considered for biomass estimations, it suffers from the limitations that i) intracellular concentrations vary up to 25-fold (10,16,33), and ii) it has been found in other archeabacteria (10,16). Quantification of unique methanogen lipids requires labor intensive extraction and analysis procedures. CoM, one of the few naturally-occurring sulfonic acids (31), was discovered (17) and identified in the mid-1970's (29). It is only found in methanogens, the concentration varies by only a factor of 5 among species (3), and the standard material is commercially available.

Previously developed techniques for the assay of CoM include a bioassay (3) and an HPLC-based method which measured fluorescent isoindole derivatives of thiols (20). The latter method was not standardized for biomass determinations (20). Both methods are cumbersome, time-consuming, require strictly anaerobic conditions, and were not designed for use with sediments.

We have modified the HPLC based procedure (20) to quantify CoM within hours of sample collection without the requirement for anoxic conditions. We have also standardized the technique with pure cultures to allow for the quantitation of methanogen biomass from a variety of environmental matrices.

Methods

Growth of Methanogens

Methanococcus (Mc.) thermolithotrophicus (DSM 2095) and *Methanococcus (Mc.) voltae* (DSM 1537) were grown as previously described (30,32). *Methanobacterium (Mb.) thermoautotrophicum* strain Marburg (DSM 2133), *Methanospirillum (Msp.) hungatei* strain GP1 (DSM 1101) and *Methanosarcina (Ms.) barkeri* strain Fusaro (DSM 804) were cultured as described (9). *Ms. barkeri* was grown on N_2/CO_2/methanol, H_2/CO_2, and N_2/CO_2/acetate. *Methanolobus (Ml.) tindarius* (DSM 2278) was grown as previously described (15) with 0.4% methanol added from a sterile stock solution. *Methanosaeta (Mst.) concilli* (DSM 3671) was grown as described (25). The MPN assays were 3-tube assays to a 10^{-8} dilution. The media formulations for MPN assays on pure methanogenic cultures were as described above for the respective organisms. For MPN assays on sediment samples, the medium used was medium 1 prepared as previously described (2). Direct cell counts were performed using a hemocytometer. All experiments were performed twice in duplicate. The data is presented as an average with standard deviation where appropriate.

Methanogen cultures (100 ml.) were grown to late log phase, harvested by centrifugation (2,700 x g; 20min; 5-10°C), and resuspended in 10ml of sterile medium. For determination of CoM over time, cultures were grown in 2 L batch cultures, and 100ml of culture was collected and harvested at periodic intervals.

Protein content for pure cultures was determined using the BCA Protein Assay (Pierce Chemical Co., USA).

HPLC Equipment and Solvent System

A Beckman Model 157 Fluorescence Detector (338nm excitation filter and 450nm emission filter) was used to quantitate CoM. HPLC analysis was accomplished using an Econosphere C18 column. The mobile phase was 50mM sodium acetate buffer (pH=5.7): acetonitrile (70:30) flowing at 1 ml/minute with a 20µl injection loop. Identification of the isoindole derivative of CoM (calculated mass=301) was achieved using an LC/MS (Hewlett-Packard Series 1100) with an Econosphere C18 column. The mobile phase was 10mM ammonium acetate buffer (pH=5.7): acetonitrile (70:30) flowing at 1ml/min. Both a standard containing 100µM CoM and an extract of hydrocarbon contaminated sediment were tested and analyzed in the electrospray negative mode for the mass spectrophotometer and scanned from 100-500 mass units. The retention time for CoM with both solvent systems was tested using synthetic CoM.

Analysis of Co-enzyme M and Samples

All reagents were purchased from Sigma and standards and stock solutions were prepared fresh daily. A 1mM CoM stock solution was made by dissolving CoM in 50mM acetate/1mM EDTA buffer, pH 4.5. All dilutions were made into 1% tri-(N)-butylphosphine in 2-propanol (1%-TBP). The reagents used for the derivatization of CoM were 20µl each of *o*-phthalaldehyde (20mg/ml methanol)

and ethanolamine (20□l/ml boric acid buffer pH 9.0) per ml sample (15). Standards were allowed to derivatize aerobically for 5min at room temperature. A variety of thiols were tested to aid in whether common thiols would interfere with CoM quantitation. Each was made as a 1mM stock solution and diluted into 1%-TBP prior to derivatization.

The linearity of the assay was determined by diluting the 1mM CoM stock solution into 1%-TBP. The concentration of the standards ranged from 0.1 μM-100 μM; corresponding to 2-2000 pmoles of CoM injected.

For extraction of CoM from cells, 0.5ml of cells were mixed with 1ml of 1%-TBP and incubated in eppendorf tubes for 1h at room temperature. Samples were then centrifuged (12,000 x g; 2min). The supernatant (1ml) was derivatized as described above and analyzed by HPLC.

The effect of freezing on the extractability of CoM was tested with cultures of *Ms. barkeri* (acetate grown), *Mc. thermolithotrophicus*, *Mc. voltae*, and *Mb. thermoautotrophicum* Marburg. Cultures were harvested, with aliquots immediately assayed for CoM as above or stored frozen at -60°C for 3d in eppendorf tubes. After 3d, samples were thawed at room temperature, and assayed as described above.

A variety of environmental matrices were sampled and assayed for CoM. These included sediments from a landfill leachate-contaminated aquifer (1,5,6) a hydrocarbon contaminated aquifer (4,7,8,28), a campus pond, as well as sewage sludge. For analysis of environmental samples, 5g were mixed with 2ml of 1%-TBP and incubated (1h) at room temperature to extract CoM.

To maximize solvent recovery, the sediment was weighed and placed into a 10ml syringe with its plunger removed and a piece of Whatman filter paper placed in the bottom of the syringe barrel. The syringe was set into a hole cut in the lid of a capped 50ml centrifuge tube (Nalgene). After centrifugation (27,000 x g), the syringe and sediment were discarded and 1ml of the supernatant within the centrifuge tube was assayed as described above.

The adsorption of CoM to sediments was tested by addition of 100µl of 0.1 - 50 µM (2 - 1000 pmoles) CoM into sediment that had been previously determined to contain no detectable CoM. The CoM spiked sediment was incubated aerobically at room temperature (3h) followed by extraction and derivatization as described above.

To determine the completeness of the CoM extraction, environmental samples were subjected to an additional extraction. Sediment samples from the leachate impacted aquifer and pond sediment were assayed for CoM, and re-extracted and assayed again.

The effect of freezing on CoM extractability in environmental samples was tested. Landfill aquifer sediment, hydrocarbon contaminated sediment, pond sediment, and sewage sludge (5g each) were all analyzed with an additional 5g set aside and frozen at -60°C for 3d in stoppered 10ml syringes. After 3d, samples were thawed at room temperature and CoM was quantitated.

Results

Assay Development and Validation

The HPLC method yielded an isolated peak at approximately 3.6min when the sample was in water and 2.9min when in isopropanol. That peak was confirmed to be the isoindole derivative of CoM using mass spectroscopy (fig. 1). A detection limit of 2pmoles was achieved based on the analysis of standards. In both pure methanogen cultures and environmental samples several other peaks were observed. However, there was no difficulty in resolving the CoM peak. All other thiols tested eluted at different times than CoM (Table 1). The assay proved linear with synthetic CoM (2-2000 pmoles CoM), pure methanogen cultures (0.5-5.0 ml), and environmental samples (0.5-5.0 g). Synthetic CoM, spiked into environmental samples, was almost completely recovered and mirrored the synthetic CoM regression line, indicating there was no interference from sediments and that extracellular CoM did not adsorb to sediments.

Mb. thermoautotrophicum Marburg and *Msp. hungatei* were both analyzed over the course of growth. With *Mb. thermoautotrophicum* Marburg, total CoM (CoM/ml culture) increased with time from 0.03-0.31 nmol CoM while A_{600} increased from 0.02-0.17. However when normalized for protein content, there was no significant change in CoM/mg protein over time (fig. 2). Similar results were observed with *Msp. hungatei* (data not shown). This indicated that levels of CoM/mg protein are stable and as a consequence, the growth phase of the cells will not influence CoM levels in natural samples.

CoM Content of Cultures and Sediments

Pure cultures of methanogens were compared to determine the variability in CoM levels among different organisms and findings suggest that CoM levels may vary up to 7-fold (Table 2). From the environmental samples tested, all contained measurable amounts of CoM (Table 3) with sewage sludge having the highest CoM content (on a per gram basis) at 0.09nmol CoM/gm.

Extraction Efficiency of CoM Assay

Several cultures and anoxic sediments were tested to determine whether a second extraction increased CoM recovery. For pure cultures, 87.8-96.8% of the total CoM detectable was recovered in the first extraction while data for environmental samples ranged from 91.8-100% recovery during the first extraction. The results suggest that only a single extraction is required to recover the majority of CoM.

Cell Lysis with French Press

Ms. barkeri (N_2/CO_2/methanol), *Msp. hungatei*, *Mb. thermoautotrophicum* Marburg, *Mc. thermolithotrophicus*, and *Mc. voltae* were tested to determine if cell lysis increased total CoM recovery. Cells were lysed using a french pressure cell at 38,000psi. CoM was extracted from cells and cell extracts in the usual manner. In all four methanogens tested, results were similar.

Effect of Freezing on CoM

Pure cultures and anoxic sediments were tested to determine if freezing had an effect on CoM recovery. With all pure cultures there was no significant loss of CoM after freezing (fig. 3a). In contrast to the pure cultures tested, all sediments showed a significant loss in detectable CoM after samples were frozen and thawed (fig. 3b).

Calculation of CoM per Cell

Calculation of CoM on a per cell basis was performed by both MPN assay and a direct cell counting procedure. Calculations of CoM on a per cell basis were comparable and the overall value between organisms were similar (Table 2). Determinations based on direct cell counts ranged from 0.14fmol CoM/cell in *Mb. thermoautotrophicum* to 0.70fmol CoM/cell in *Msp. hungatei* giving an average value of 0.40±0.20 fmol CoM/cell. The corresponding MPN-based determinations ranged from 0.34fmol CoM/cell in *Mc. thermolithotrophicus* to 0.47fmol CoM/cell in *Msp. hungatei*, giving an average value of 0.39±0.07 fmol CoM/cell.

Calculations of CoM per cell were made for sediment from a campus pond, a landfill leachate-contaminated aquifer, sewage sludge, and 6 different depths along a sediment core from the hydrocarbon contaminated site (Table 3). In the sediment core from the hydrocarbon contaminated site, values ranged from 0.43fmol CoM/cell to 0.74fmol CoM/cell. Values from the other 3 sediments ranged from 0.18fmol CoM/cell to 0.48fmol CoM/cell. An overall average value of 0.41±0.17 fmol CoM/cell was calculated by using the average value from the

hydrocarbon contaminated site (0.59fmol CoM/cell) with the values from the other 3 samples.

Discussion

For many applications in environmental microbiology, accurate estimations of microbial population size is beneficial. Methanogens are important in many anoxic environments and carry out the terminal electron accepting process in a variety of ecosystems. As such, an accurate estimation of methanogen biomass is often desirable.

Previously, several marine thiols including CoM were quantified using a similar HPLC-based technique involving pre-column derivatization (20). In a more recent study, this technique was used to quantitate thiols in pore waters but not in sediments (30). This is not the first report of such methodology for thiol detection. Two forms of CoM, Mesna (sodium 2-mercaptoethanesulfonate) and Dimesna (2-mercaptoethanesulfonate disulfate), were quantitated using HPLC based methods with post column derivatization and colorimetry (13,27). The difference in the post-column methods from the detection procedure used in this study is that two steps were involved; to separate the thiol of interest from others compounds, derivatization and quantitation were performed separately, thereby requiring additional labor. More recently, CoM was detected using a fluorescent precolumn derivatization procedure followed by reverse phase HPLC (11). This work is similar to the present study, but differs in that the synthesis of the derivatizing reagents was difficult and time consuming whereas the reagents required here are readily available.

We have modified the previous work (20) by the addition of 1% TBP. The 1% TBP acts as a reducing agent to prevent the oxidation of CoM-SH to a homodisulfide of CoM-S-S-CoM or a heterodisulfide of CoM-S-S-HTP (7-mercaptoheptanoylthreonine phosphate) (21,22) as the cells were lysed in isopropanol. This allows the assay to be conducted without stringent anoxic conditions. Quantification of CoM has been completed for several pure cultures of methanogens and environmental samples. The protocol can be accomplished within 3h of sample collection. Using this technique, an accurate estimate of the methanogenic population at a given site may be ascertained. The conclusion is based on several points, including a detection limit of 2pmoles CoM and the linear response of the assay for both the synthetic cofactor and CoM obtained from pure cultures. The assay was found applicable to environmental samples. An HPLC-based method for Coenzyme A quantitation (12) found similar results, suggesting that a similar technique may be developed for other organisms.

Experimentation revealed that the change in CoM concentration increased with the absorbance of pure cultures over time, but that the CoM/mg protein was constant. Thus, the CoM content per cell varied little over the growth phase. The same trend was found for coenzyme F_{420} in methanogens (26). We observed cellular concentrations of CoM/mg protein to vary by up to a factor of 7 among organisms; comparable to the factor of 5 associated with the previously published CoM bioassay (3). Values for CoM/mg protein were consistent in the current study as compared to the bioassay (3). *Ms. barkeri* (grown on methanol) showed 41.1nmol CoM/mg protein compared to 44.4nmol CoM/mg protein as reported in

the bioassay while *Mb. thermoautotrophicum* was found to have 7.5nmol CoM/mg protein as compared to 6.7nmol CoM/mg protein reported in the bioassay. *Msp. hungatei* was not as consistent with 19.4nmol CoM/mg protein as opposed to the 3.9nmol CoM/mg protein reported previously (3).

While there was no loss of CoM detectability in pure cultures due to freezing, environmental samples showed a 46-83% decrease. While the reasons for this are not clear, such findings suggest that it is not advisable to store environmental samples in the freezer prior to the assay.

Comparison of CoM biomass determinations using the HPLC-based assay with direct cell counts or MPN assays agreed well. Average values of 0.40 ± 0.20 fmol CoM/cell with direct cell counts and 0.39 ± 0.07 fmol CoM/cell with MPN were attained for pure cultures, and 0.41 ± 0.17 fmol CoM/cell for environmental samples. The similarity of values in calculating CoM/cell using MPN data for environmental samples indicates that a reference value for methanogens is possible. This would allow quick and accurate estimation of methanogen populations directly from the HPLC based CoM values. From the data obtained in this study, there is at most a 3-fold difference for CoM/cell in environmental samples. Also, using a reference value of 0.41fmol CoM/cell, the laborious MPN assay would no longer be required, allowing for a reasonably accurate estimation of the methanogen population in the time required to complete the assay.

The inherent advantage of this procedure over previous work is that CoM content per cell has been determined for a variety of environmental matrices. This

procedure provides a quick and simple aerobic method for detection of the co-factor as a biomarker and thus quantitation of methanogen biomass.

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Table 1: Retention Times of Various Thiols using the HPLC Procedure

Compound	Retention Time (minutes)
Coenzyme M	2.90
Thiophenol	3.40
1-octanethiol	3.46
Tioglycolate	3.90
Thiosulfate	3.97
Glutathione	4.10
Heptylmercaptan	4.92
Mercaptosuccinic acid	6.53
Thiosalycilic acid	7.05
2-bromoethanesulfonic acid	8.49
1-pentanethiol	8.96
2-mercaptoethanol	10.63
Cyclopentylmercaptan	10.78

Table 2: Distribution of Coenzyme M Levels in Various Methanogens and Determination of Coenzyme M Levels per Cell

	CoM		Direct Counts		MPN Estimation	
Organism	nmol/mg protein	nmol/ml	cells/ml	fmol CoM/cell	cells/ml	fmol CoM/cell
Ml. tindarius	11.49	0.56				
Msp. hungatei	19.41	0.52	7.40×10^5	0.70	1.1×10^6	0.47
Ms. barkeri (grown on N_2/CO_2/methanol)	41.11	1.97	4.60×10^6	0.43		
Ms. barkeri (grown on N_2/CO_2/acetate)	6.13	0.70	1.80×10^6	0.39		
Ms. barkeri (grown on H_2/CO_2)	18.21	1.78				
Mst. concilli	6.59	0.38				
Mc. voltae	23.75	1.45	6.20×10^6	0.23		
Mc. thermolithotrophicus	41.09	2.59	5.15×10^6	0.50	7.5×10^6	0.34
Mb. thermoautotrophicum Marburg	7.48	0.40	2.85×10^6	0.14	1.1×10^7	0.36

Table 3: Distribution of Coenzyme M Levels in Various Sediments and Determination of Coenzyme M Levels per Cell for Sediments

		CoM		MPN Estimation	
Environmental Sample		(nmol/g sediment)	(nmol/ml)	(cells/ml)	(fmol CoM/cell)
Pond Sediment		0.02	0.06	$1.5x10^5$	0.40
Landfill Sediment		0.01	0.02	$1.1x10^5$	0.18
Sewage Sludge		0.09	0.22	$4.6x10^5$	0.48
Hydrocarbon Contaminated Sediment					
--various depths	2.5 ft.	0.00	ND	ND	-----
	3.0 ft.	0.02	0.05	$7.5x10^4$	0.67
	4.0 ft.	0.08	0.20	$3.3x10^5$	0.47
	5.5 ft.	0.07	0.18	$2.2x10^5$	0.65
	7.5 ft.	0.08	0.20	$3.6x10^5$	0.43
	8.5 ft.	0.07	0.19	$2.7x10^5$	0.55
	10.0 ft.	0.07	0.17	$1.6x10^5$	0.74

ND - not determined

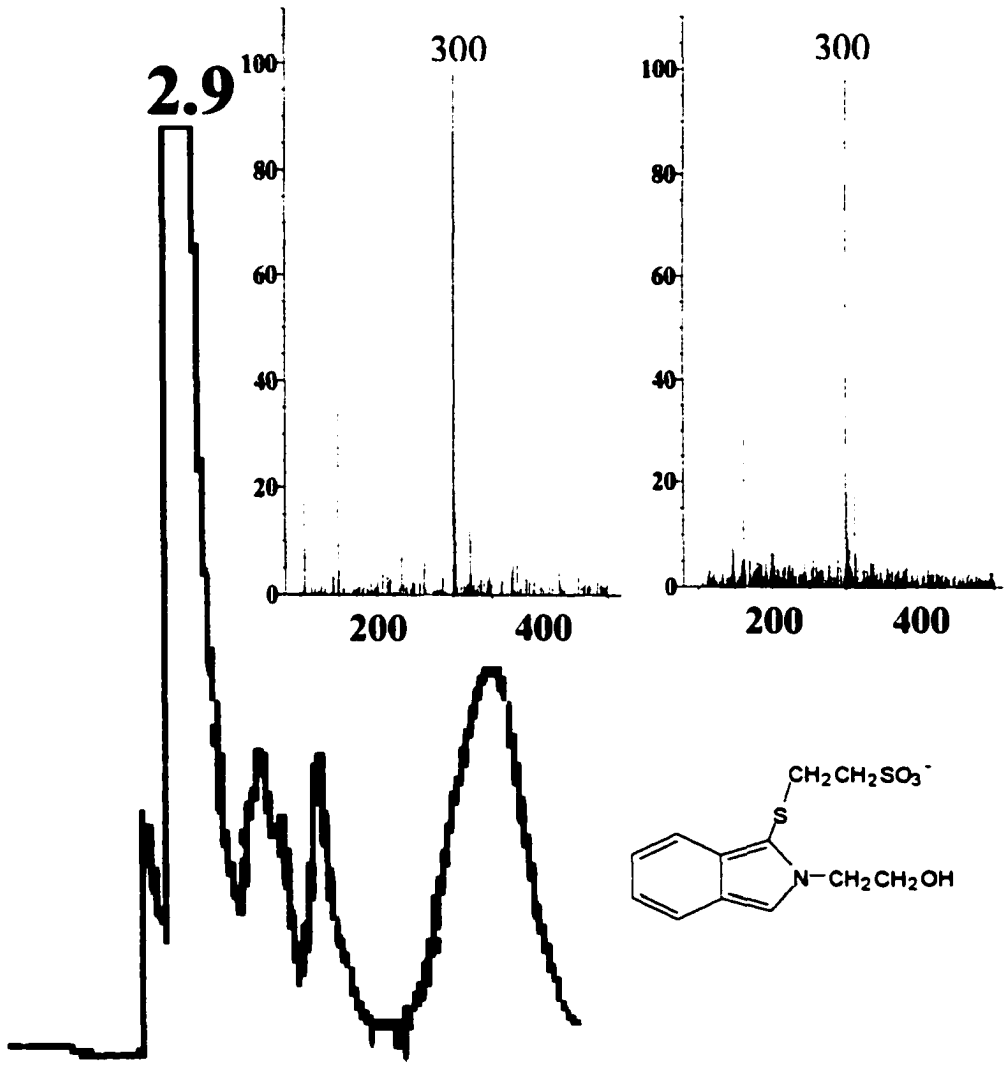

Figure 1: HPLC chromatogram of derivatized CoM (R.T.= 2.9 minutes) from the hydrocarbon contaminated aquifer. The buffer system used was ammonium acetate: acetonitrile (70:30) in both the HPLC and the LC/MS. The inset compares the LC/MS profile of the isoindole derivative of standard CoM (left) with the corresponding peak from the environmental sample (right). Both spectra show an ionic molecular weight of 300 which is consistent with the structure (left inset) of the fluorescent CoM derivative.

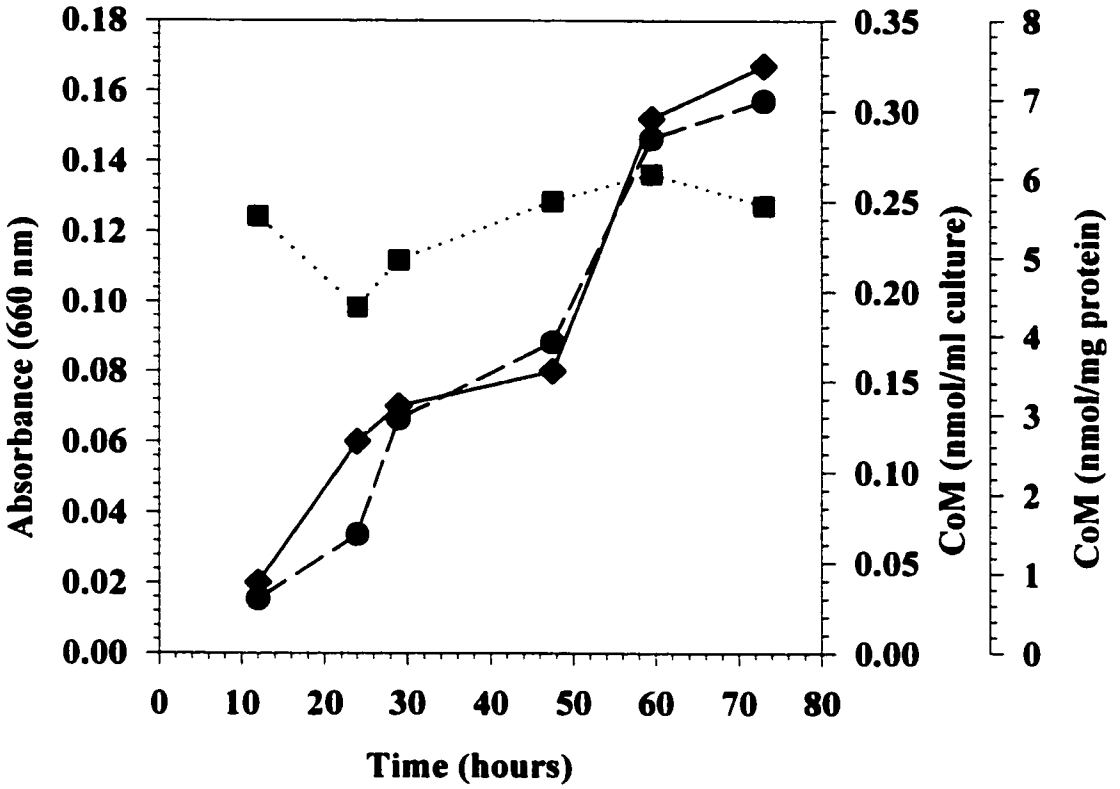

Figure 2: Absorbance (●), CoM content/ml culture (◆), and CoM normalized for culture protein (■) in a *Methanobacterium thermoautotrophicum* Marburg culture while growing on hydrogen.

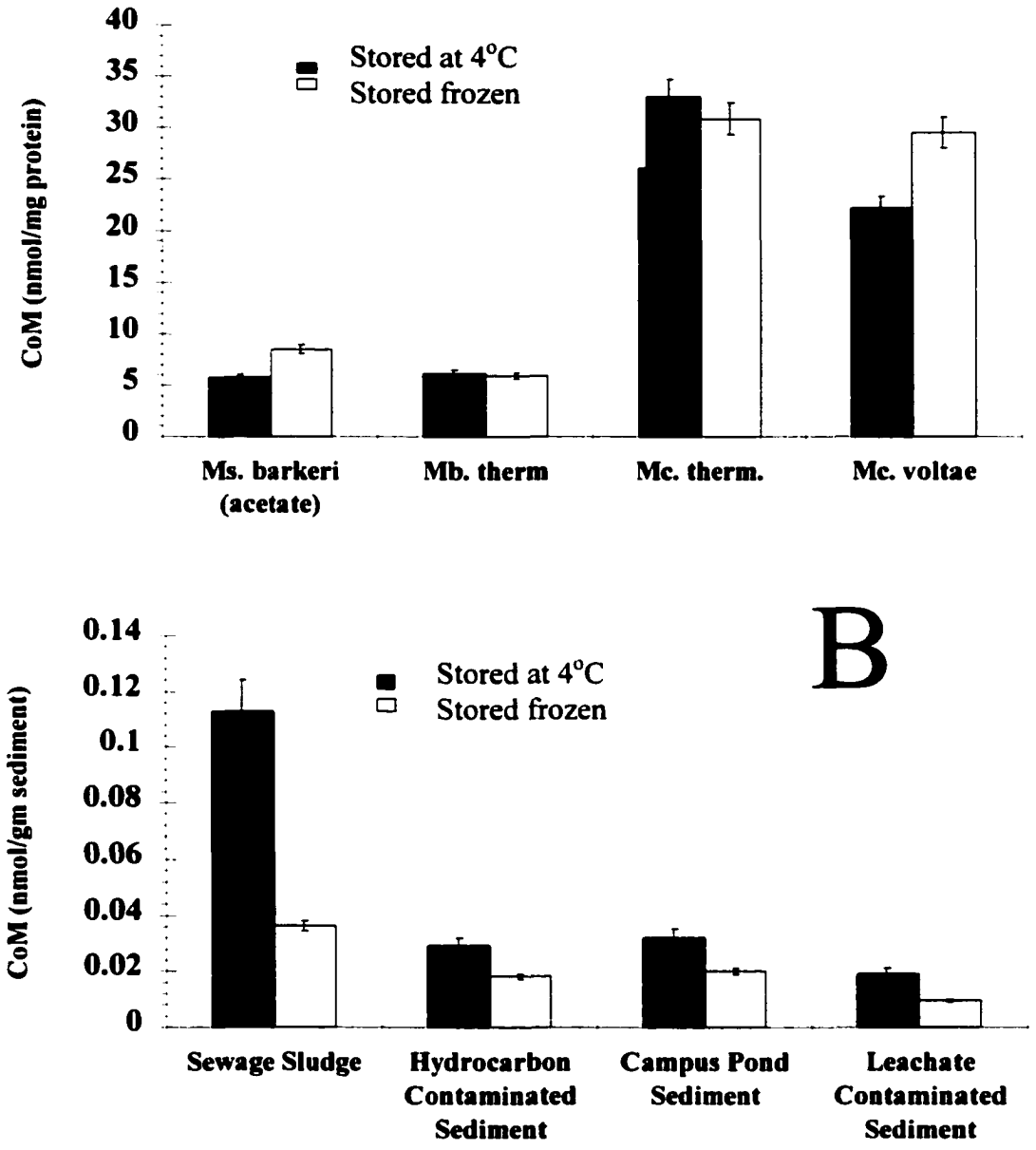

Figure 3: (A) Cultures of methanogens were tested before and after freezing at –60°C. There was no loss of CoM detection in cells due to freezing. (B) Environmental samples were tested before and after freezing at –60°C. There was a loss of 46-83% of detectable CoM depending on the matrix. Bars indicate standard error.