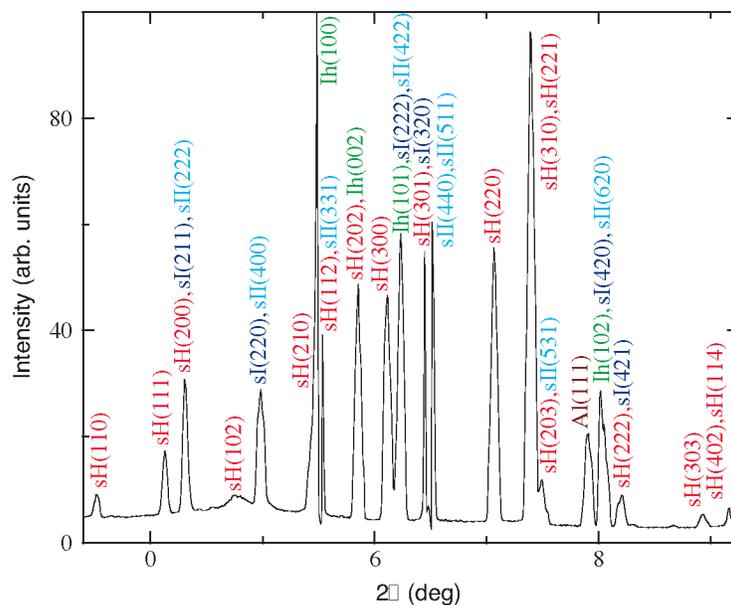
The background of the entire page is a close-up, slightly blurred image of the United States flag, showing the stars and stripes. A large, semi-transparent watermark of the Naval Research Laboratory seal is centered over the flag. The seal features an eagle with wings spread, a shield on its chest, and a cross. The text "UNITED STATES OF AMERICA" is at the top and "NAVAL RESEARCH LABORATORY" is at the bottom of the seal's border.

2002

NRL REVIEW

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**FIGURE 10** X-ray diffraction of natural hydrate collected from the Gulf of Mexico. Measurement performed at 500 psi and 150 K using 33 keV (0.3757 Å) photons from the Advanced Photon Source. Diffraction peaks labeled from hydrate structure H (red), structure I (blue), and structure II (cyan), and water ice lh (green).

and university facilities throughout the United States, Canada, Norway, Japan, Korea, and Russia address topics in future energy, ocean floor fuel cell development, coastal stability, ocean carbon cycling, and global warming.

**Acknowledgments:** The NRL research program is an Advance Research Initiative, “Alteration of Sediment Properties Through Dissociation of Gas Hydrates.” Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Science.

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## STUDY OF MICROBIAL CHROMIUM(VI) REDUCTION BY ELECTRON ENERGY LOSS SPECTROSCOPY

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**Introduction:** The geochemistry and toxicity of chromium (Cr) are controlled by valence state. Chromium is a redox active 3d transition metal with a wide range (−2 to +6) of possible oxidation states, of

which only two are stable. Thermodynamic calculations predict that soluble Cr(VI) is energetically favored for oxic conditions, while insoluble Cr(III) is favored under anoxic or suboxic conditions. Hexavalent chromium species are strong oxidants that act as carcinogens, mutagens, and teratogens in biological systems. Therefore, microbial Cr(VI) reduction is of particular technological and biological importance because it converts a toxic, mobile element into a less toxic, immobile form.

Study of microbial Cr(VI) reduction, such as identification of reduction intermediates, has been hindered by the lack of analytical techniques that can identify oxidation state with subcellular spatial resolution. The most common method for measuring Cr(VI) reduction in bacterial cultures is the diphenylcarbazide colorimetric assay in which Cr(VI) concentration is determined from absorbance at 540 nm by the stoichiometric oxidation products of diphenylcarbazide reagent. However, this bulk technique cannot provide the submicron-scale information necessary for understanding microbial reduction processes. One technique with sufficient spatial resolution is electron energy loss spectroscopy (EELS). EELS directly measures the energy loss of incident electrons that inelastically scatter from atoms in the specimen and is a direct probe of the electron configuration around atoms. Consequently, EELS can identify the oxidation state of 3d and 4d transition metals.<sup>1</sup> Despite the detailed, submicron-scale infor-

mation EELS techniques can provide on oxidation state, they have never been applied in microbial reduction studies. This article demonstrates the application of EELS for the determination of metal oxidation state in studies of microbial reduction. Specifically, we examined reduction of Cr(VI) in anaerobic cultures of *Shewanella oneidensis* containing Cr(VI)O<sub>4</sub><sup>2-</sup>. *S. oneidensis* is a gram-negative, facultative bacterium, capable of respiring aerobically and anaerobically by using a variety of terminal electron acceptors.<sup>2</sup> It is a member of the  $\gamma$ -subclass of *Proteobacteria*, and has been isolated from lacustrine and marine environments.

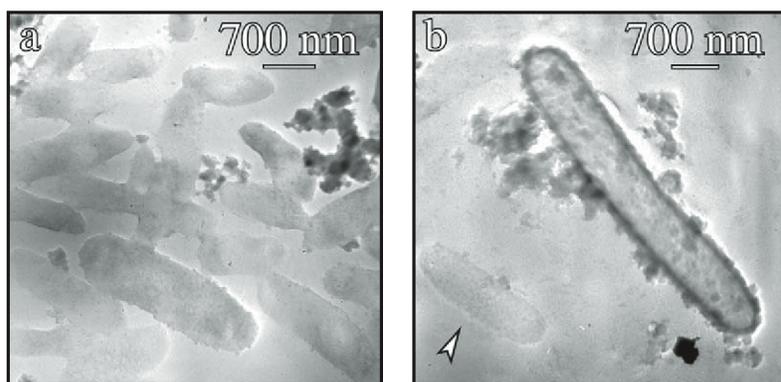
**Methodology:** Determination of oxidation state by EELS is accomplished by analyzing valence-induced differences in fine structure of L<sub>2</sub> and L<sub>3</sub> (or collectively L<sub>2,3</sub>) absorption edges. The L<sub>2,3</sub> absorption edges arise from transitions to unoccupied *d* levels from two spin-orbit split levels: the 2*p*<sub>1/2</sub> level (producing the L<sub>2</sub> edge) and the 2*p*<sub>3/2</sub> level (producing the L<sub>3</sub> edge). The valence of a transition metal is related to the number of holes in the *d* level, i.e., the 3*d*<sup>*n*</sup> (or 4*d*<sup>*n*</sup>) configuration. For example, tetrahedral Cr(VI) has an empty *d* orbital (3*d*<sup>0</sup> configuration) and octahedral Cr(III) has a 3*d*<sup>3</sup> configuration. Since L<sub>2,3</sub> absorption edges are inherently dependent on the number of unoccupied *d* levels in 3*d* and 4*d* transition metals, they are sensitive to valence state.

Bacterial cultures were examined directly by environmental cell (EC)-transmission electron microscopy (TEM) at 100 Torr, under a circulation of air saturated with water vapor. The EC-TEM system is of the closed-cell type. A pressurized environment is maintained by two electron-transparent, amorphous-carbon windows with the specimen supported on the lower window. Bacteria were also examined in cross section by conventional TEM after embedding and thin sectioning.

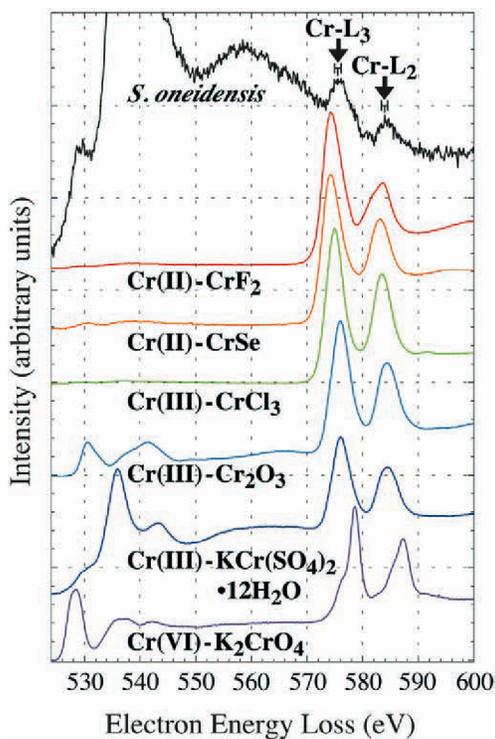
**Results:** Examination by EC-TEM shows the typical rod-shaped morphology of *S. oneidensis*. In particular, the bacterial membranes are intact and do not show evidence of rupture by partial decompression. Cells remain plump/hydrated, while extracellular polymeric substances encapsulating the cells retain moisture. Electron microscopy reveals two distinct populations of *S. oneidensis* in incubated cultures containing Cr(VI): cells that exhibit low image contrast (Fig. 11(a)) and heavily precipitate-encrusted cells that exhibit high image contrast (Figs. 11(b)).

Several EELS techniques were applied to determine the oxidation state of Cr associated with the encrusted cells. Oxidation state was determined by measuring the chemical shift and intensity ratios of Cr-L<sub>2,3</sub> adsorption peaks.<sup>3</sup> Figure 12 compares the EELS spectra of encrusted, hydrated *S. oneidensis* collected by EC-TEM to that of Cr oxidation-state standards collected by conventional TEM. The correlation between measured L<sub>3</sub>/L<sub>2</sub> integrated-peak intensity ratios and L<sub>3</sub> peak positions for standards demonstrates that different oxidation states fall within well-separated regions (Fig. 13). Within a given oxidation state, spectra of individual standards fall within separate groupings, reflecting possible differences in atom coordination, spin-orbit interactions, and crystal field splitting. Comparison with the standards demonstrates that precipitate-encrusted bacteria contain Cr in oxidation state +3 or lower (Fig. 13). Precipitates encrusting bacteria were also examined in cross section. EELS measurements by conventional TEM of cross sections (Fig. 13) are consistent with measurements of encrusted, hydrated bacteria by EC-TEM, demonstrating that EELS provides accurate data, even under the more onerous experimental conditions of the EC.

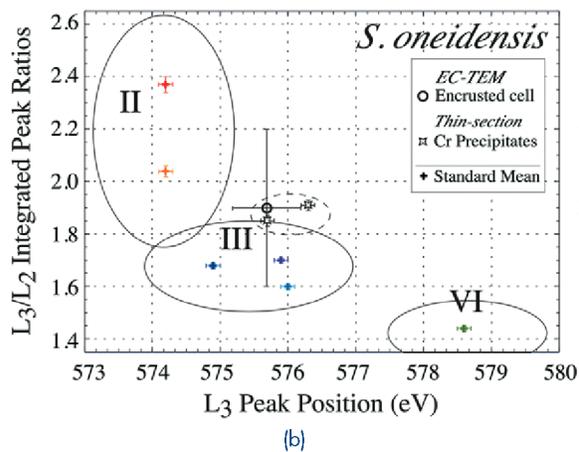
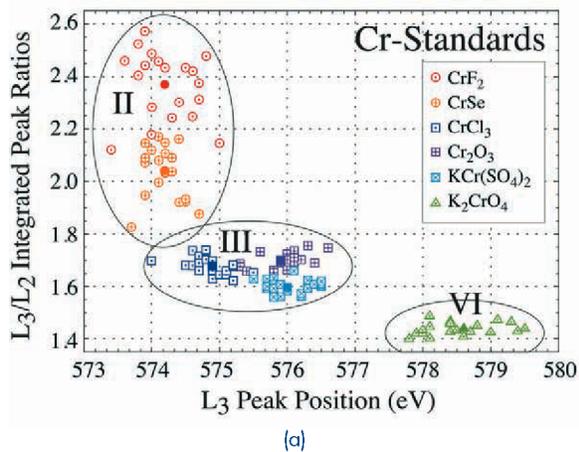
**Summary:** Chemical and oxidation state information for the microbial reduction of Cr(VI) by the



**FIGURE 11**  
*Shewanella oneidensis* imaged by EC-TEM at 100 Torr: bacteria (a) exhibiting low contrast and (b) encrusted with electron dense precipitates. Arrowhead in (b) points to a low contrast bacterium, illustrating the dramatic contrast difference with respect to encrusted bacteria.



**FIGURE 12**  
Comparison of EELS spectra of encrusted *Shewanella oneidensis* in the EC and Cr oxidation-state standards. Spectra were normalized to the intensity of the L<sub>3</sub> peak and offset from one another.



**FIGURE 13**  
Correlation between measured L<sub>3</sub>/L<sub>2</sub> integrated-peak ratios and L<sub>3</sub> peak positions (a) Cr oxidation-state standards, (b) bacteria and precipitates (solid data points represent the mean of the data for a particular Cr standard).

facultative anaerobe *Shewanella oneidensis* was acquired with high spatial resolution using EELS. We demonstrate that quantitative measurements of oxidation state can be performed on hydrated specimens by EC-TEM. This is the first time the oxidation state of microbial metal-reduction products, localized with bacteria, has been measured. Such information is vital for identifying microbial electron transfer sites and transfer mechanisms.

[Sponsored by ONR]

#### References

- <sup>1</sup> R.F. Egerton, *Electron Energy-loss Spectroscopy in the Electron Microscope* (Plenum, New York, 1996).
- <sup>2</sup> K. Venkateswaran, D.P. Moser, M.E. Dollhopf, D.P. Lies, D.A. Saffarini, B.J. MacGregor, D.B. Ringelberg, D.C. White, M. Nishijima, H. Sano, J. Burghardt, E. Stackebrandt, and K.H. Nealson, "Polyphasic Taxonomy of the Genus *Shewanella* and Description of *Shewanella oneidensis* sp. nov.," *Int. J. Syst. Bacteriol.* **49**, 705-724 (1999).
- <sup>3</sup> T.L. Daulton, B.J. Little, K. Lowe, and J. Jones-Meehan, "In-situ Environmental Cell-Transmission Electron Microscopy Study of Microbial Reduction of Chromium(VI) using Electron Energy Loss Spectroscopy," *Microscopy Microanal.*, in press. ■