

MOLECULAR ASPECTS OF URANIUM TOXICITY:
SPECIATION AND PHYSIOLOGICAL TARGETING

by

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ABSTRACT

Uranium (U), as the uranyl ion (UO_2^{2+}), is a widely distributed contaminant at several Department of Energy (DOE) sites, former war zones, and across the globe. Although many U remediation efforts depend on U-bacterial interactions, little information regarding U-bacterial interactions resolved at the molecular level exist. In this study, experiments were performed aimed at understanding the effect of molecular UO_2^{2+} speciation on bacterial bioaccumulation and toxicity using an environmental *Pseudomonas* sp. isolate. Results showed that the charge and stability of UO_2^{2+} species largely controlled the extent of UO_2^{2+} bioaccumulation and UO_2^{2+} toxicity, respectively. Further experimentation, including a combination of *in vivo*, *in vitro*, and *in silico* studies, revealed a specific mechanism of UO_2^{2+} toxicity, the first to be reported. This mechanism involves the binding of UO_2^{2+} to pyrroloquinoline quinone (PQQ), a cofactor present in a number of bacterial dehydrogenase enzymes. Based on the specific binding mode of UO_2^{2+} to PQQ, it is hypothesized that the present work has direct implications for UO_2^{2+} inhibition of flavoproteins, potentially extending the application of the findings of this work to eukaryotic systems. Recent trends suggest that U-related activity will increase in the near future, and therefore understanding fundamental interactions between UO_2^{2+} and living systems is both an environmental and human health imperative.

ENVIRONMENTALLY RELEVANT URANIUM USES, CHEMISTRY, CONTAMINATION, AND REMEDIATION

Introduction

Uranium (U) is a toxic metal found naturally in the earth's crust and oceans, and at a number of U contaminated sites in locations dotting the globe. These sites pose significant human health and environmental threats, and research originally devoted to pure U chemistry have been applied by those seeking to understand both the fate and mobility of U contamination, and how this information might be used in UO_2^{2+} remediation efforts. This chapter seeks to highlight environmentally relevant aspects of U, including its production and uses, its chemistry, and how these relate to U contamination and remediation.

Uranium Sources and Principle Uses

Uranium (U), the heaviest naturally occurring element on earth, ranks 51st among the elements in terms of crustal abundance, where it is present at an average concentration of 2.7 mg-U/kg (75). In the oceans, it is present at relatively uniform concentrations of 3.3 $\mu\text{g-U/L}$ (36).

In general, U minerals are either comprised mostly of hexavalent U(VI) or reduced U(IV), although some mixed-valence minerals do exist (13). There are many more examples of U(VI)-containing minerals than U(IV)-containing minerals, owing somewhat to the higher crystallinity of U(IV) minerals compared to the complex structures of U(VI) minerals (13). However, reduced U(IV) minerals are the most

common constituent of many U deposits. The most common U(IV) mineral is uraninite (UO_2), an insoluble form of U that has been given much attention due to its implications in U fate and mobility in contaminated environments, a topic to be discussed in a later section.

The vast majority of uranium produced is currently used as fuel for nuclear reactors, which supplied 5.8% of the world's energy consumption in 2007 (11). Canada is the leading producer of U, followed by Australia and Kazakhstan (76). The United States (US) ranks 8th among U producing countries, with virtually all mining activity taking place in the western part of the country, and in Colorado, Utah, and Wyoming in particular (78) (Figure 1).

Uranium Contamination and Remediation

Improper handling and storage of U has led to the widespread distribution of U contamination of soil and groundwater. U contamination in the United States (US) is largely associated with radioactive legacy waste generated from the development of nuclear weaponry during World War II and the Cold War, and remains a persistent problem at a number of Department of Energy (DOE) sites, including Pacific Northwest National Laboratory (PNNL) (12, 39) and Oak Ridge National Laboratory (4). However, U contamination has resulted from a number of other activities, both military and civilian. The use of depleted uranium (DU) in armor-piercing ammunition was officially introduced in 1991 during the first Gulf War conflict, and has contributed to global U contamination, particularly in former war zones. These include Kosovo (47, 66) where

30,000 DU rounds were fired during the 1999 conflict (79), and Bosnia and Herzegovina where 10,800 DU rounds were fired during the 1994 conflict (38). A number of DU munitions testing sites also house significant amounts of contamination (24, 58). The DU munitions plant in Colonie, near Albany, New York, is currently surrounded by a DU plume in which U concentrations exceed 300 ppm (1.3 mM), a concentration exceeding EPA drinking water standards by a factor of 10,000 (60).

Private sector industrial and mining activity has also led to the release of substantial amounts of U into the environment. The Old Rifle site is a former uranium-ore processing facility in Rifle, CO, (USA) currently part of the Uranium Mill Tailings Remedial Action (UMTRA) program initiated by the DOE (77, 83). Decades of TiO_2 production and U extrusion processes by the RMI Titanium Company in the Fields Brook watershed has led to U contamination in sediments of the Ashtabula River in Ohio, US (35). A bog near Concord, MA is currently designated as a priority site by the Massachusetts Department of Environmental Protection due to the presence of elevated levels of DU originating from a leaking holding basin located at a nuclear industrial facility (17).

As mentioned earlier, U is naturally found in either the U(VI) or U(IV) oxidation state. This has implications for U fate and mobility at contaminated sites as U(VI) exhibits greater mobility due to its solubility while reduced U, U(IV), forms relatively insoluble uraninite precipitates (UO_2) and therefore exhibits less environmental mobility (64, 84). In addition, U(IV) is considered less toxic than U(VI) (81). The reduction of U(VI) contaminants to U(IV) has therefore been studied as an option for remediation

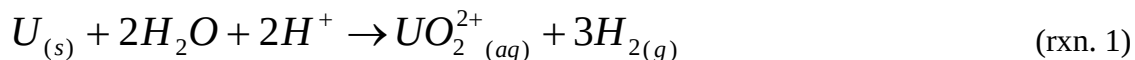
efforts. In particular, the ability of bacteria to facilitate this reduction has given them an important place among U remediation technologies (1, 15). Bacterial U(VI) reduction to U(IV) is generally considered an anaerobic process and is usually seen under iron-, nitrate-, or sulfate-reducing conditions facilitated by iron reducing (85), denitrifying (18, 72), and sulfate reducing bacteria (40, 65).

While bacterial U(VI) reduction under anaerobic conditions has been an important process influencing U mobility at a number of contaminated sites, there are other microbe-metal interactions which could be incorporated into bioremediation strategies (37). In particular, the influence bacteria have under aerobic conditions has received relatively little attention to date, but interest is growing given the number of contaminated sites in which aerobic conditions prevail (7, 45). These include contaminated vadose zone soils, conditions found, for example, at the DOE Field Research Center (FRC) in Oak Ridge, Tennessee (74), and at the Drigg Low Level Waste (LLW) site in Cumbria, North West England (21). U contaminated, unsaturated soils can also be found at a number of low level waste (LLW) sites in the United States, including the Idaho National Laboratory (INL), located in southern Idaho (31, 48, 49), the Beatty site south of Beatty, Nevada, the Los Alamos National Laboratory, Los Alamos, New Mexico (9) and the Richland site, located on the DOE's Hanford Reservation in southeastern Washington State (54, 86). Under aerobic conditions, U(VI) reduction is not expected to play a significant role in overall U mobility due to the tendency of U(IV) to reoxidize in the presence of oxygen (52, 53).

Bacterial bioaccumulation, which can have a significant effect under aerobic conditions, has been studied in laboratory settings as a means of immobilizing toxic metals including cadmium, lead, copper, zinc, and U(VI) (82). Researchers also investigated this phenomenon as a potential component of bioremediation schemes in aerobic systems (3, 41-43, 61, 80). Among the factors that influence bioaccumulation are the conditions under which bacteria are grown, including choice of pH buffer and carbon source (14, 29, 67). For example, U(VI) accumulation has been shown to be slower in cells growing in rich media compared to minimal media (73). Understanding these factors, especially as they relate to contaminated sites, is therefore important for understanding environmentally relevant U-microbe interactions under aerobic conditions.

Uranium Aqueous Chemistry

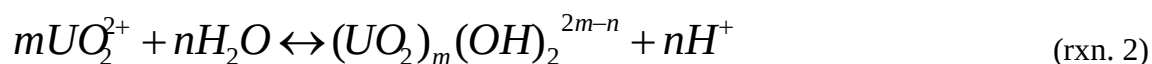
Elemental uranium is not found naturally as it corrodes readily in the presence of water and atmospheric oxygen to produce the hexavalent uranyl cation (U(VI)O_2^{2+}) and hydrogen gas (H_2) (19, 59). Although this reaction proceeds through a number of intermediates, it can be summarized according to reaction 1 (27):



The uranyl ion is a dioxo-cation in which two oxygen atoms are bound axially to the U(VI) atom (Figure 2). Typically these bonds lengths are close to 180 pm (68), and this

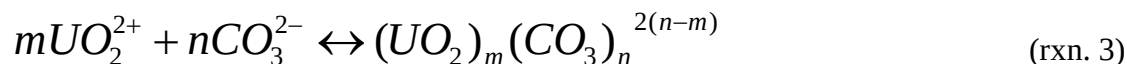
length has been used to suggest that the oxo-uranium bonds exhibit third order character, and that the effective charge on the U(VI) is +3 (16).

Complexation of UO_2^{2+} occurs in the equatorial plane perpendicular to the axial oxygen atoms, and UO_2^{2+} is usually bound by four, five, or six ligands (13). Among the best studied aqueous UO_2^{2+} complexes are hydrolysis products and carbonate complexes, since they form in all aqueous systems exposed to CO_2 , and therefore are of particular environmental and human health significance. In general, hydrolysis products form according to reaction 2 (26):



Typical values of m range from 1 to 4, and n can range from 1 to 7 (see Figure 3).

Similarly, uranyl carbonate complexes are formed according to reaction 3 (26):



In these systems, m is usually either 1 or 2, and n can range from 1 to 3 (see Figure 3). In

addition, mixed complexes containing both carboxyl hydroxyl groups can form (46)

(reaction 4):



The distribution of these species is highly pH dependent. Generally, acidic conditions favor the presence of uncomplexed UO_2^{2+} . This can be explained by considering the stoichiometries of reactions 2-4. High H^+ concentrations will drive the equilibrium of reaction 2 toward uncomplexed UO_2^{2+} . Since carbonic acid (H_2CO_3) dissociates at $\text{pH} \approx 6.3$ (56), CO_3^{2-} will largely be absent at low pH conditions and therefore will not contribute significantly to UO_2^{2+} speciation under acidic conditions. The pH dependence of UO_2^{2+} complexation and speciation is shown in Figure 3, generated using MINTEQ (ver. 2.52). In agreement with previously published speciation diagrams (55), the lowest pH conditions are dominated by free UO_2^{2+} , which are replaced between pH values of 5 and 6 as the major species by uranyl hydrolysis products. Above $\text{pH} = 6$, UO_2^{2+} complexation by carbonate begins to dominate, first as a mixed complex $((\text{UO}_2)_2\text{CO}_3(\text{OH})^-)$, and then as the tris-carbanato species $\text{UO}_2(\text{CO}_3)_3^{4-}$. This last complex ($\text{UO}_2(\text{CO}_3)_3^{4-}$) is of particular importance as it is believed to be the dominant UO_2^{2+} species *in vivo* (30).

Uranyl phosphates, which have the general formula $(\text{UO}_2)_r(\text{PO}_4)_q^{2+r-3q}$ (26), are another class of important complexes, given the presence of phosphates both in the environment and in biological systems. Typical values for r range from 0-2, and q can vary between 1 and 3. In open, aqueous systems, uranyl phosphate complexes form at pH values between 0 and 6, and tend to replace the free uranyl ion and uranyl hydrolysis products which would otherwise form in the absence of PO_4^{3-} (2). However, at $\text{pH} = 6$ uranyl carbonate complexes begin to form and at $\text{pH} = 7$ dominate UO_2^{2+} speciation. Unlike carbonate and hydroxyl complexes, uranyl phosphates more readily form

insoluble species (44). The ability of PO_4^{3-} to form uranyl precipitates has been utilized in U remediation schemes (34), and also by bacteria as a detoxification mechanism (42, 71).

In addition to inorganic complexants, a number of organic molecules will readily chelate UO_2^{2+} , particularly those with carboxyl groups including acetate, citrate, and lactate (23). Ethylenediamine triacetic acid (EDTA) and nitrilotriacetic acid (NTA) are also commonly used UO_2^{2+} chelators which produce highly stable complexes. Uranyl also readily binds to nitrogen donating ligands, including 8-hydroxyquinoline, which, when used to modify Amberlite resin can be used as an efficient means of concentrating UO_2^{2+} from seawater (70). Recent work has emphasized the ability of bacterial siderophores to bind UO_2^{2+} (51). However, little is known about UO_2^{2+} binding to important biomolecules, including proteins, peptides, cofactors, and nucleotides (81). Therefore, expanding our understanding of UO_2^{2+} complexation to include such molecules would represent an important advancement in UO_2^{2+} biological chemistry and would possibly lead to a better understanding of UO_2^{2+} toxicity.

In addition to understanding biologically relevant UO_2^{2+} binding, it is important to recognize the stability of various uranyl complexes and their relationship to bioavailability. In general, stable UO_2^{2+} species, including uranyl carbonate complexes, are less toxic to microorganisms, and in some cases are reduced to U(IV)O_2 at a slower rate (23). Unstable species, including uranyl hydrolysis products, tend to exhibit more toxic effects to cells, both prokaryotic and eukaryotic (20, 22, 28, 33, 63). Table 1 provides a summary of common UO_2^{2+} complexes and their stability constants, information which can often be used to estimate the relative level of toxicity experienced

by organisms growing under a given set of conditions (28). For example, conditions which promote the presence of unstable uranyl hydroxide complexes are expected to lead to a higher level of toxicity relative to conditions that favor the presence of stable uranyl carbonate species. For example, *Pseudomonas* spp. are much more sensitive to UO_2^{2+} toxicity when speciation is dominated by unstable hydroxide complexes than when grown in the presence of stable uranyl carbonate complexes (8).

Hypotheses Regarding the Influence of Cellulosic Breakdown on Uranium Toxicity to Bacteria and Bacterial Bioaccumulation

Understanding how uranium complexation and speciation influences U mobility and U toxicity to native organisms is relevant to low level waste (LLW) sites, including the one present at the Idaho National Laboratory (INL) discussed in Section 1.3. LLW sites house contaminated cellulosic materials, including paper towels, mop heads, and lab coats. These materials, and specifically their breakdown products, potentially contribute to both UO_2^{2+} immobilization and mobilization. For example, these breakdown products include short-chain fatty acids, such as acetic and lactic acids, which can potentially enhance UO_2^{2+} mobility through chelation (5, 6, 10, 50, 62). Mechanisms of UO_2^{2+} immobilization include UO_2^{2+} bioaccumulation within bacterial biomass metabolizing the cellulose and cellulosic breakdown products (25, 57, 69). It was hypothesized that these products would significantly influence the UO_2^{2+} bioaccumulation potential of a growing bacterial culture. Specifically, it was hypothesized that UO_2^{2+} toxicity to the culture would vary significantly depending on the cellulose breakdown product being utilized. This would strongly influence the total accumulation potential in the system, as this is

directly related to the total number of cells in the system. It was also hypothesized that the presence of cellulose breakdown products would influence UO_2^{2+} accumulation on a per-cell basis, in addition to the final cell concentration reached in the system. Finally, it was hypothesized that UO_2^{2+} speciation, particularly as it relates to bioavailability, would be sufficient to explain observed toxicity and bioaccumulation patterns. These hypotheses were investigated, and the results of these studies are presented in the following chapter.

The overarching goal of this dissertation is to investigate molecular aspects of UO_2^{2+} toxicity. This was accomplished by first explaining general UO_2^{2+} toxicity patterns in terms of UO_2^{2+} speciation, as detailed in Chapter 2. Efforts were then focused on understanding UO_2^{2+} toxicity as a product of cellular physiology, with the goal of elucidating specific UO_2^{2+} binding modes causing acute UO_2^{2+} toxicity as discussed in Chapters 3 and 4. The results discussed in this dissertation provide valuable information relating UO_2^{2+} speciation and toxicity, and also introduce the first mechanism of UO_2^{2+} toxicity resolved at the molecular level.

Table 1. Stability constants of various UO_2^{2+} complexes mentioned in the text, assuming standard state ($T = 298.15 \text{ K}$, $P = 0.1 \text{ MPa}$) and infinite dilution ($I = 0$). Values taken from Guillaumont, et al. (2003) and Hummel, et al. (2005). $K = [\text{UO}_2\text{L}]/[\text{UO}_2^{2+}][\text{L}]$, where $[\text{UO}_2^{2+}]$ is the UO_2^{2+} concentration at equilibrium, $[\text{L}]$ is ligand concentration at equilibrium, and $[\text{UO}_2\text{L}]$ is the metal complex at equilibrium (Guillaumont, et al., 2003).

Ligand	log K
Hydroxide (OH^-)	-5.25
Carbonate (CO_3^{2-})	9.9
Phosphate (PO_4^{3-})	13.2
Acetate ($\text{C}_2\text{H}_3\text{O}_2^-$)	4.6
Lactate ($\text{C}_3\text{H}_5\text{O}_3^-$)	2.9
Citrate ($\text{C}_6\text{H}_5\text{O}_7^{3-}$)	9.0
EDTA [$\text{CH}_2\text{N}(\text{CH}_2\text{CO}_2)_2$] $_2^{4-}$	13.7
NTA [$\text{N}(\text{C}_2\text{H}_2\text{O}_2)$] $_3^{3-}$	10.8

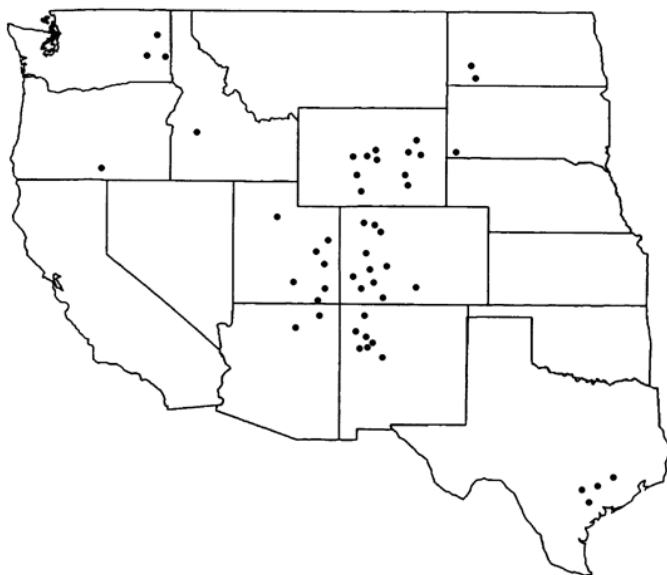


Figure 1. Map of U mine locations in the western US. Dots correspond to specific mining sites.

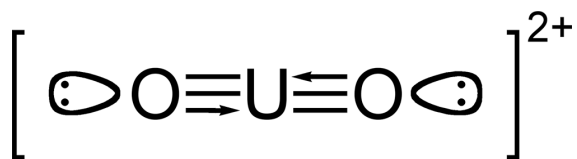


Figure 2. Dioxo-uranyl cation showing third order bonding between uranium atom and axial oxygens.

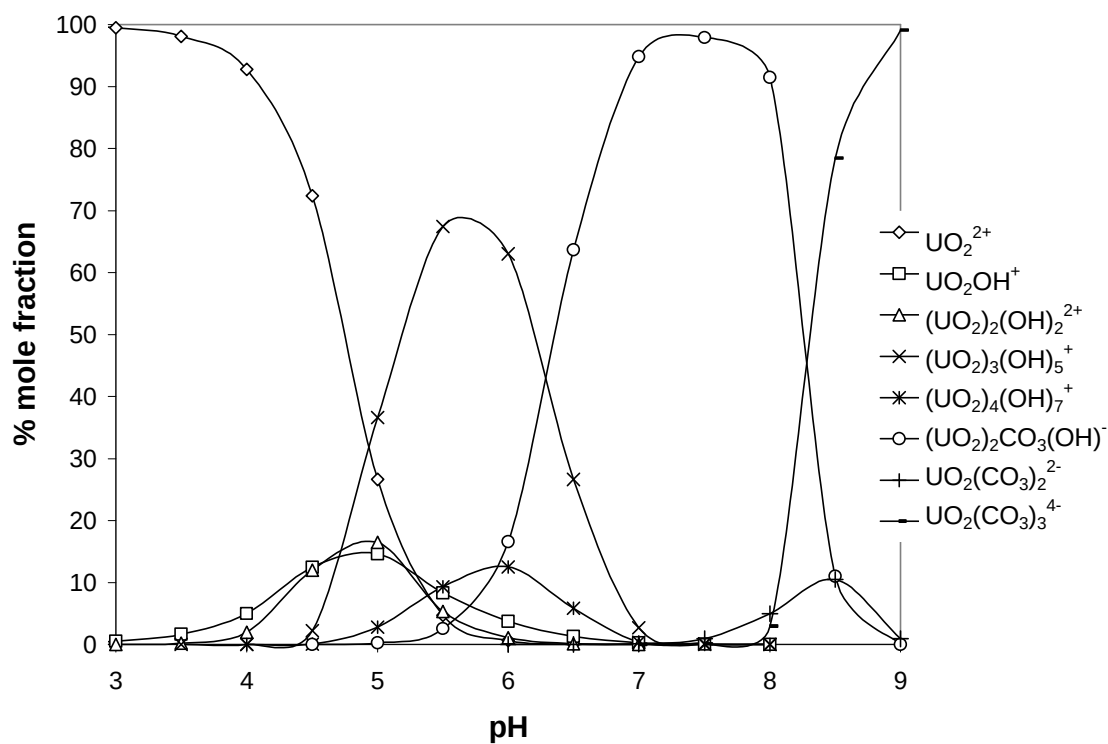


Figure 3. Distribution of dominant uranyl species as a function of pH at 25°C with $[\text{UO}_2^{2+}] = 50 \mu\text{M}$ and aqueous CO_3^{2-} in equilibrium with atmospheric CO_2 ($P_{\text{CO}_2} = 38.5 \text{ Pa}$). Data points were calculated using MINTEQ (ver. 2.52).

References

1. Anderson RT, Vrionis HA, Ortiz-Bernad I, Resch CT, Long PE, Dayvault R, Karp K, Marutzky S, Metzler DR, Peacock A, White DC, Lowe M, Lovley DR. 2003. Stimulating the in situ activity of *Geobacter* species to remove uranium from the groundwater of a uranium-contaminated aquifer. *Appl. Environ. Microbiol.* 69(10): 5884-5891.
2. Ansoborlo E, Prat O, Moisy P, Den Auwer C, Guilbaud P, Carriere M, Gouget B, Duffield J, Doizi D, Vercouter T, Moulin C, Moulin V. 2006. Actinide speciation in relation to biological processes. *Biochimie.* 88:1605-1618.
3. Appukuttan D, Rao AS, Apte SK. 2006. Engineering of *Deinococcus radiodurans* R1 for Bioprecipitation of Uranium from Dilute Nuclear Waste. *Appl. Environ. Microbiol.* 72(12):7873-7878.
4. Bagwell CE, Liu X, Wu L, Zhou J. 2006. Effects of legacy nuclear waste on the compositional diversity and distributions of sulfate-reducing bacteria in the terrestrial subsurface groundwater. *FEMS Microbiol. Ecol.* 55:424-431.
5. Baston GMN, Berry JA, Bond KA, Brownsword M, Linklater CM. 1992. Effects of Organic Degradation Products on the Sorption of Actinides. *Radiochim. Acta.* 58/59:349-356.
6. Baston GMN, Berry JA, Bond KA, Boulton KA, Brownsword M, Linklater CM. 1994. Effects of cellulosic degradation products on uranium sorption in the geosphere. *J. Alloy. Compound.* 213/214:475-480.
7. Beazley MJ, Martinez RJ, Sobecky PA, Webb SM, Taillefer M. 2007. Uranium Biomineralization as a Result of Bacterial Phosphatase Activity: Insights from Bacterial Isolates from a Contaminated Subsurface. *Environ. Sci. Tech.* 41:5701-5707.
8. Bencheikh-Latmani R, Leckie O. 2003. Association of uranyl with the cell wall of *Pseudomonas fluorescens* inhibits metabolism. *Geochim. Cosmochim. Acta.* 67(21): 4057-4066.
9. Birdsell KH, Wolfsberg AV, Hollis D, Cherry TA, Bower KM. 2000. Groundwater flow and radionuclide transport calculations for a performance assessment of a low-level waste site. *J. Contam. Hydrol.* 46:99-129.

10. Bourbon X, Toulhoat P. 1996. Influence of Organic Degradation Products on the Solubilisation of Radionuclides in Intermediate and Low Level Radioactive Wastes. *Radiochim. Acta.* 74:315-319.
11. British Petroleum. 2009. *BP Statistical Review of World Energy, June 2009*. Retrieved from:
http://www.bp.com/liveassets/bp_internet/globalbp/globalbp_uk_english/reports_and_publications/statistical_energy_review_2008/STAGING/local_assets/2009_downloads/statistical_review_of_world_energy_full_report_2009.pdf
12. Brooks SC, Fredrickson JK, Carroll SL, Kennedy DW, Zachara JM, Plymale AE, Kelly SD, Kemner KM and Fendorf S. 2003. Inhibition of Bacterial U(VI) Reduction by Calcium. *Environ. Sci. Technol.* 37:1850-1858.
13. Burns PC. 1999. *Uranium: mineralogy, geochemistry, and the environment*. Mineralogical Society of America.
14. Chang J-S, Law R, Chang C-C. 1997. Biosorption of Lead, Copper, and Cadmium by Biomass of *Pseudomonas aeruginosa* PU21. *Wat. Res.* 31(7):1651-1658.
15. Chang YJ, Long PE, Geyer R, Peacock AD, Resch CT, Sublette K, Pfiffner S, Smithgall A, Anderson RT, Vrionis HA, Stephen JR, Dayvault R, Ortiz-Bernad I, Lovley DR, White DC. 2005. Microbial incorporation of C-13-labeled acetate at the field scale: Detection of microbes responsible for reduction of U(VI). *Environ. Sci. Technol.* 39(23):9039-9048.
16. Choppin GR, Rao LF. 1984. *Radiochim. Acta.* 37:143.
17. Edmands JD, Brabander DJ, Coleman DS. 2001. Uptake and mobility of uranium in black oaks: implications for biomonitoring depleted uranium-contaminated groundwater. *Chemosphere.* 44:789-795.
18. Edwards L, Kusel K, Drake H, Kostka JE. 2007. Electron flow in acidic subsurface sediments co-contaminated with nitrate and uranium. *Geochim Cosmochim Acta.* 71(3):643-654.
19. Fonnesebeck JE. 2000. Quantitative analysis of hydrogen gas formed by aqueous corrosion of metallic uranium. Argonne National Laboratory – West, Idaho Falls, ID.
20. Fortin C, Dutel L, Garnier-Laplace J. 2004. Uranium complexation and uptake by a green alga in relation to chemical speciation: the importance of the free uranyl ion. *Environ. Toxicol. Chem.* 23(4):974-981.

21. Fox JR, Mortimer RJG, Lear G, Lloyd JR, Beadle I, Morris K. 2006. The biogeochemical behaviour of U(VI) in the simulated near-field of a low-level radioactive waste repository. *Appl. Geochem.* 21:1539-1550.
22. Franklin NM, Stauber JL, Markich SJ, Lim RP. 2000. pH-dependent toxicity of copper and uranium to a tropical freshwater alga (*Chlorella* sp.). *Aquat. Toxicol.* 48:275-289.
23. Ganesh R, Robinson KG, Reed GD, Sayler GS. 1997. Reduction of Hexavalent Uranium from Organic Complexes by Sulfate- and Iron-Reducing Bacteria. *Appl. Environ. Microb.* 63(11):4385-4391.
24. Giannardi C, Dominici, D. 2003. Military use of depleted uranium: assessment of prolonged population exposure. *J. Environ. Radioact.* 64:227-236.
25. Gorman-Lewis D, Elias PE, Fein JB. 2005. Adsorption of Aqueous Uranyl Complexes onto *Bacillus subtilis* Cells. *Environ Sci Techol* 39:4906-4912.
26. Guillaumont R, Fanghänel T, Fuger J, Grenthe I, Neck V, Palmer DA, Rand MH. 2003. *Chemical thermodynamics Volume 5: Update on the chemical thermodynamics of Uranium, Neptunium, Plutonium, Americium, and Technetium*. Elsevier B. V., Amsterdam, The Netherlands.
27. Haschke JM. 1998. Corrosion of uranium in air and water vapor: consequences for environmental dispersal. *J. Alloy. Compound.* 278:149-160.
28. Hogan AC, van Dam RA, Markich SJ, Camilleri C. 2005. Chronic toxicity of uranium to a tropical green alga (*Chlorella* sp.) in natural waters and the influence of dissolved organic carbon. *Aquat. Toxicol.* 75:343-353.
29. Hu MZ-C, Reeves M. 1997. Biosorption of Uranium by *Pseudomonas aeruginosa* Strain CSU Immobilized in a Novel Matrix. *Biotechnol. Prog.* 13:60-70.
30. Huang H, Chaudhary S, Van Horn JD. 2005. Uranyl-Peptide Interactions in Carbonate Solution with DAHK and Derivatives. *Inorg. Chem.* 44:813-815.
31. Hull LC, Grossman C, Fjeld RA, Coates JT, Elzerman AW. 2004. Hybrid empirical-theoretical approach to modeling uranium adsorption. *Appl. Geochem.* 19:721-736.
32. Hummel W, Anderegg G, Rao L, Puigdomenech I, Tochiyama O. 2005. *Chemical thermodynamics of compounds and complexes of U, Np, Pu, Am, Tc, Ni and Zr with selected organic ligands*. NEA-OECD, Elsevier B. V., Amsterdam, The Netherlands.

33. Hyne RV, Rippon GD, Ellender G. 1992. pH-Dependent uranium toxicity to freshwater hydra. *Sci. Tot. Environ.* 125:159-173.
34. Jerden JL, Sinha AK. 2003. Phosphate based immobilization of uranium in an oxidizing bedrock aquifer. *Appl. Geochem.* 18:823-843.
35. Ketterer ME, Wetzel WC, Layman RR, Matisoff G, Bonniwell EC. 2000. Isotopic studies of Sources of Uranium in Sediments of the Ashtabula River, Ohio, U.S.A. *Environ. Sci. Technol.* 34:966-972.
36. Klinkhammer GP, Palmer MR. 1991. Uranium in the oceans: Where it goes and why. *Geochim. Cosmochim. Acta.* 55:1799-1806.
37. Kumar R, Singh S, Singh OV. 2007. Bioremediation of radionuclides: Emerging technologies. *OMICS-A J. Integrat. Biol.* 11(3):295-304.
38. Lind OC, Salbu B, Skipperud L, Janssens K, Jaroszewicz J, De Nolf W. 2009. Solid state speciation and potential bioavailability of depleted uranium particles from Kosovo and Kuwait. *J. Environ. Radioact.* 100:301-307.
39. Liu C, Gorby YA, Zachara JM, Fredrickson JK, Brown CF. 2002. Reduction Kinetics of Fe(III), Co(III), U(VI), Cr(VI), and Tc(VII) in Cultures of Dissimilatory Metal-Reducing Bacteria. *Biotechnol. Bioeng.* 80:637-649.
40. Luo W, Wu W-M, Yan T, Criddle CS, Jardine PM, Zhou J, Gu B. 2007. Influence of bicarbonate, sulfate, and electron donors on biological reduction of uranium and microbial community composition. *Appl. Microbiol. Biot.* 77:713-721.
41. Macaskie LE, Blackmore JD, Empson RM. 1988. Phosphatase overproduction and enhanced uranium accumulation by a stable mutant of a *Citrobacter* sp. isolated by a novel method. *FEMS Microbiol. Lett.* 55:157-162.
42. Macaskie LE, Empson RM, Cheetham AK, Grey CP, Skarnulis AJ. 1992. Uranium Bioaccumulation by a *Citrobacter* sp. as a Result of Enzymically Mediated Growth of Polycrystalline HUO_2PO_4 . *Science.* 257:782-784.
43. Macaskie LE, Bonthron KM, Rouch DA. 1994. Phosphatase-mediated heavy metal accumulation by a *Citrobacter* sp. and related enterobacteria. *FEMS Microbiol. Lett.* 121:141-146.
44. Marković M, Pavcović N. 1983. Solubility and Equilibrium Constants of Uranyl(2+) in Phosphate Solutions. *Inorg. Chem.* 22:978-982.

45. Martinez RJ, Beazley MJ, Taillefert M, Arakaki AK, Skolnick J, Sobecky PA. 2007. Aerobic uranium (VI) bioprecipitation by metal-resistant bacteria isolated from radionuclide- and metal-contaminated subsurface soils. *Environ. Microbiol.* 9(12): 3122-3133.
46. Maya L. 1982. Electrochemical and sorption characteristics of hydroxo and carbonato species of dioxo uranium-VI. *Abstracts of Papers of the American Chemical Society.* 183:11.
47. Milačić S, Petrović D, Jovičić D, Kovačević R, Simić J. 2004. Examination of the health status of populations from depleted-uranium-contaminated regions. *Environ. Res.* 95:2-10.
48. Mincher BJ, Fox RV, Cooper DC, Groenewold GS. 2003a. Neptunium and plutonium sorption to Snake River Plain, Idaho soil. *Radiochim. Acta.* 91:397-401.
49. Mincher BJ, Fox RV, Riddle CL, Cooper DC, Groenewold GS. 2003b. Strontium and cesium sorption to Snake River Plain, Idaho soil. *Radiochim. Acta.* 91:1-7.
50. Moll H, Geipel G, Reich T, Bernhard G, Fanghänel T, Grenthe I. 2003. Uranyl(VI) complexes with alpha-substituted carboxylic acids in aqueous solution. *Radiochim. Acta.* 91:11-20.
51. Moll H, Glorius M, Bernhard G, Johnsson A, Pedersen K, Schäfer M, Budzikiewicz H. 2008. Characterization of Pyoverdins Secreted by a Subsurface Strain of *Pseudomonas fluorescens* and Their Interactions with Uranium(VI). *Geomicrobiol. J.* 25:157-166.
52. Moon HS, Komlos J, Jaffe PR. 2007. Uranium Oxidation in Previously Bioreduced Sediment by Dissolved Oxygen and Nitrate. *Environ. Sci. Technol.* 41:4587-4592.
53. Moon HS, Komlos J, Jaffe PR. 2009. Biogenic U(IV) oxidation by dissolved oxygen and nitrate in sediment after prolonged U(VI)/Fe(III)/SO₄²⁻ reduction. *J. Contaminant Hydrology.* 105:18-27.
54. Moser DP, Fredrickson JK, Geist DR, Arntzen EV, Peacock AD, Li S-MW, Spadoni T, McKinley JP. 2003. Biogeochemical Processes and Microbial Characteristics across Groundwater – Surface Water Boundaries of the Hanford Reach of the Columbia River. *Environ. Sci. Tech.* 37:5127-5134.
55. Moulin C, Decambox P. 1995. Uranium Speciation in Solution by Time-Resolved Laser-Induced Fluorescence. *Anal. Chem.* 67:348-353.

56. Nakayama F. 1970. Sodium bicarbonate and carbonate ion pairs and their relation to estimation of first and second dissociation constants of carbonic acid. *J. Phys. Chem.* 74(13):2726-2728.
57. Nancharaiah YV, Joshi HM, Mohan TVK, Venugopalan VP, Narasimhan SV. 2006. Aerobic granular biomass: a novel biomaterial for efficient uranium removal. *Curr. Sci.* 91:503-509.
58. Oliver IW, Graham MC, MacKenzie AB, Ellam RM, Farmer JG. 2008. Distribution and partitioning of depleted uranium (DU) in soils at weapons test ranges – Investigations combining BCR extraction scheme and isotopic analysis. *Chemosphere.* 72:932-939.
59. Peretrukhin VF, Maslennikov AG, Tsivadze AY, Delegard CH, Yusov AB, Shilov VP, Bessonov AA, German KE, Fedoseev AM, Kazanskii LP, Budanova NY, Kareta AV, Gogolev AV, Gedgovd KN, Bulatov GS. 2008. Corrosion of Uranium and Its Low Content Zr, Nb, and Ru Alloys in Aqueous Solutions. *Protect. Metal.* 44(3):211-232.
60. Parrish RR, Horstwood M, Arnason JG, Chenery S, Brewer T, Lloyd NS, Carpenter DO. 2008. Depleted uranium contamination by inhalation exposure and its detection after ~20 years: Implications for human health assessment. *Sci. Tot. Environ.* 390:58-68.
61. Powers LG, Mills HJ, Palumbo AV, Zhang C, Delaney K, Sobecky PA. 2002. Introduction of a plasmid-encoded *phoA* gene for constitutive overproduction of alkaline phosphatase in three subsurface *Pseudomonas* isolates. *FEMS Microbiol. Ecol.* 41:115-123.
62. Read D, Ross D, Sims RJ. 1998. The migration of uranium through Clashach Sandstone: the role of low molecular weight organics in enhancing radionuclide transport. *J. Contam. Hydrol.* 35:235-248.
63. Riethmuller N, Markich SJ, Van Dam RA, Parry D. 2001. Effects of water hardness and alkalinity on the toxicity of uranium to a tropical freshwater hydra (*Hydra viridissima*). *Biomark.* 6(1):45-51.
64. Roden EE, Scheibe TD. 2005. Conceptual and numerical model of uranium(VI) reductive immobilization in fractured subsurface sediments. *Chemosphere.* 59:617-628.
65. Sani RK, Peyton BM, Dohnalkova A. 2006. Toxic Effects of Uranium on *Desulfovibrio desulfuricans* G20. *Environ. Toxicol. Chem.* 35(5):1231-1238.

66. Sansone U, Danesi PR, Barbizzi S, Belli M, Campbell M, Gaudino S, Jia G, Ocone R, Pati A, Rosamilia S, Stellato L. 2001. Radioecological survey at selected sites hit by depleted uranium ammunitions during the 1999 Kosovo conflict. *Sci. Tot. Environ.* 281:23-35.
67. Sar P, D'Souza SF. 2001. Biosorptive uranium uptake by a *Pseudomonas* strain: characterization and equilibrium studies. *J. Chem. Technol. Biotechnol.* 76:1286-1294.
68. Schlosser F, Krüger S, Rösch N. 2006. A Density Functional Study of Uranyl Monocarboxylates. *Inorg. Chem.* 45:1480-1490.
69. Selenska-Pobell S, Panak P, Miteva V, Boudakov I, Bernhard G, Nitsche H. 1999. Selective accumulation of heavy metals by three indigenous *Bacillus* strains, *B. cereus*, *B. megaterium* and *B. sphaericus*, from drain waters of a uranium waste pile. *FEMS Microbiol. Ecol.* 29:59-67.
70. Singh BN, Maiti B. 2006. Separation and preconcentration of U(VI) on XAD-4 modified with 8-hydroxyquinoline. *Talanta.* 69:393-396.
71. Sivaswamy, V. 2005. Uranium immobilization by *Cellulomonas* sp. ES6. Thesis, M. S. in Chemical Engineering. Washington State University, Pullman, WA, USA.
72. Spain AM, Peacock AD, Istok JD, Elshahed MS, Najar FZ, Roe BA, White DC, Krumholz LR. 2007. Identification and isolation of a *Castellaniella* species important during biostimulation of an acidic nitrate- and uranium-contaminated aquifer. *Appl. Environ. Microbiol.* 73(15):4892-4904.
73. Strandberg GW, Shumate II SE, Parrott Jr JR. 1981. Microbial Cells as Biosorbents for Heavy Metals: Accumulation of Uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 41(1):237-245.
74. Stubbs JE, Elbert DC, Veblen DR, Zhu C. 2006. Electron microbeam investigation of uranium-contaminated soils from Oak Ridge, TN, USA. *Environ. Sci. Technol.* 40(7): 2108-2113.
75. Taylor SR. 1964. Abundance of chemical elements in the continental crust: a new table. *Geochim. Cosmochim. Acta.* 28:1273-1285.
76. United Nations Statistics Database. 2007. *Energy Statistics Yearbook 2004*. United Nations, Department of Economic and Social Affairs.

77. United States Department of Energy. 1999. Final site observational work plan for the UMTRA project Old Rifle site GJO-99-88-TAR. United States Department of Energy, Grand Junction, Colorado.
78. United States Nuclear Regulatory Commission. 2002. Radioactive Waste: Production, Storage, Disposal (NUREG/BR-0216, Rev. 2). Retrieved from: <http://www.nrc.gov/reading-rm/doc-collections/nuregs/brochures/br0216/r2/mill-tailings-sites.html>, accessed 05/22/09.
79. United Nations Environmental Programme. 2001. *Depleted Uranium in Kosovo: Post-Conflict Environmental Assessment*.
80. Valls M, de Lorenzo V. 2002. Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. *FEMS Microbiol. Rev.* 26:327-338.
81. Van Horn JD, Huang H. 2006 Uranium(VI) bio-coordination chemistry from biochemical, solution and protein structural data. *Coordinat. Chem. Rev.* 250:765-775.
82. Volesky B, Holan ZR. 1995. Biosorption of Heavy Metals. *Biotechnol. Prog.* 11:235-250.
83. Vrionis HA, Anderson RT, Ortiz-Bernad I, O'Neill KR, Resch CT, Peacock AD, Dayvault R, White DC, Long PE, Lovley DR. 2005. Microbiological and geochemical heterogeneity in and in situ uranium bioremediation field site. *Appl. Environ. Microbiol.* 71(10):6308-6318.
84. Wall JD, Krumholz LR. 2006 Uranium Reduction. *Ann. Rev. Microbiol.* 60:149-166.
85. Wilkins MJ, Livens FR, Vaughan DJ, Lloyd JR. 2006. The impact of Fe(III)-reducing bacteria on uranium mobility. *Biogeochem.* 78(2):125-150.
86. Wilshire HG, Friedman I. 1999. Contaminant migration at two low-level radioactive waste sites in arid western United States – A review. *Environ. Geol.* 37(1-2):112-123.

UO₂²⁺ SPECIATION DETERMINES URANIUM TOXICITY AND
BIOACCUMULATION IN AN ENVIRONMENTAL *PSEUDOMONAS* SP. ISOLATE

Introduction

Uranium (U) is a widely distributed subsurface contaminant at several Department of Energy (DOE) sites (1), and across the globe (2). At sites where molecular oxygen is present, most U in the aqueous phase will be in the form of the hexavalent uranyl cation (UO₂²⁺). Low-level waste (LLW) sites, including the Idaho National Laboratory (Idaho Falls, ID) (3) and the Drigg site (Cumbria, NW England) (4), are among U-contaminated sites housing buried contaminated cellulosic material, such as paper towels, cardboard, and lab coats. These materials, and specifically their breakdown products, can potentially contribute to both UO₂²⁺ immobilization and mobilization. For example, these breakdown products include short-chain fatty acids which can potentially enhance UO₂²⁺ mobility through chelation (5-7). Mechanisms of UO₂²⁺ immobilization include UO₂²⁺ accumulation within biomass metabolizing the cellulose and cellulosic breakdown products (8,9,10). In its reduced valence state (U(IV)), uranium can readily precipitate as UO₂, which exhibits limited mobility and toxicity (11,12). The ability of iron- and sulfur-reducing bacteria to couple oxidation of organic substrates to U(VI) reduction under anaerobic conditions plays a prominent role in many uranium bioremediation schemes (13). However, many LLW sites are aerobic, including the INL and Drigg sites (3,4), and therefore UO₂²⁺ reduction, an anaerobic process, is not expected to play a large role in decreasing uranium mobility in these systems.

Bacterial bioaccumulation, which could potentially contribute to UO_2^{2+} immobilization, has been studied extensively in laboratory settings as a means of immobilizing toxic metals including cadmium, lead, copper, zinc, and UO_2^{2+} (14). A considerable amount of research has investigated this phenomenon as a potential component of bioremediation schemes in aerobic systems (15-17). Among the factors that have been shown to influence bioaccumulation are the conditions under which the bacteria are grown, including choice of buffer and carbon source (18,19).

Understanding the impact such growth conditions have on UO_2^{2+} bacterial bioaccumulation processes in LLW sites is important, given the range of carbon and energy sources made available during cellulosic breakdown (20). In addition, understanding how these conditions affect UO_2^{2+} toxicity is equally important because bioaccumulation will only affect UO_2^{2+} mobility significantly if native bacteria are able to reach appreciable numbers in the U-contaminated system. The purpose of this study was to investigate how select cellulosic breakdown products, as primary carbon and energy sources, affect UO_2^{2+} bioaccumulation and toxicity in a *Pseudomonas* sp. isolate (hereafter referred to as isolate A) cultured from the Cold Test Pit South (CTPS) at the INL. Previous studies have concluded that UO_2^{2+} toxicity and bioaccumulation potential is strongly influenced by complexation and speciation of the UO_2^{2+} , (21,22), and therefore, when possible, differences in toxicity and bioaccumulation patterns were associated with changes in UO_2^{2+} speciation.

Materials and Methods

Media Composition and Growth Conditions

Isolate A was isolated from the CTPS at the INL by repeated re-streaking of an aerobic liquid enrichment on agar methylcellulose (0.1%) plates. Cells were grown from frozen (-80°C) glycerol stocks (20% v/v) at $20^{\circ} \pm 0.5^{\circ}\text{C}$ in chemically defined liquid media (pH = 7.0) consisting of simulated INL groundwater (23) containing the following (per L): 1.0 mg KCl, 12.7 mg Na_2SO_4 , 3.5 mg CaO, 1.1 mg MgSO_4 , 0.825 g NH_4Cl , 0.261 g K_2HPO_4 , amended with 5 mL of Wolfe's vitamin solution (24), and 1 mL SL-4 trace elements solution (24). Unamended medium was sterilized by autoclaving at 121°C for 25 min, and the vitamin and metal solutions were filter sterilized (0.2 μm). Since cellulose breakdown can release a variety of organic molecules which can potentially serve as carbon and electron sources for native bacteria (20), representative carbon substrates were chosen from four general categories: butyrate as a model carboxylate, ethanol as an alcohol, dextrose as a sugar, and lactate, a fatty acid commonly used in studies involving UO_2^{2+} -microbe interactions. Carbon sources were added to a concentration of 15 mM-carbon. Carbon dioxide (CO_2) is the ultimate cellulosic breakdown product, which in contaminated systems would exist in equilibrium with a number of bicarbonate species in the aqueous phase. While the presence of bicarbonates is expected regardless of metabolic activity, due to equilibrium between the aqueous phase and atmospheric CO_2 , the CO_2 generated by the activity of the native bacteria could potentially lead to an excess of bicarbonate species in the system. The influence of excess bicarbonate was therefore investigated by repeating each growth experiment with

10 mM NaHCO₃ added to the media, making eight possible growth conditions. Media with added NaHCO₃ is referred to as high bicarbonate media, and media in equilibrium with atmospheric CO₂ as low bicarbonate media. Media was buffered with 10 mM of piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES).

Uranium Toxicity Experiments and Modeling

The sensitivity of isolate A to UO₂²⁺ under each of eight growth conditions was investigated. Cells were grown in the same medium to be tested and allowed to reach late exponential growth phase. Cells were washed (three cycles of centrifugation at 6,000 × g for 20 min) and suspended in 50 mL of fresh medium. Growth in high bicarbonate media took place in 125 mL serum bottles sealed with butyl stoppers and crimped with aluminum seals. The headspace was replaced regularly to prevent oxygen depletion. Growth in low bicarbonate media took place in 250 mL baffled shaker flasks. UO₂²⁺ concentrations (as uranyl chloride, UO₂Cl₂, International Bio-Analytical Industries Inc.) ranged from 0 – 250 µM. Cultures were shaken at 100 rpm at 20° ± 0.5°C. Liquid samples were periodically removed to measure protein concentrations using the Bradford assay (25). UO₂²⁺ inhibition of growth was modeled using the following generalized Monod expression (Eqn. 1) (27):

$$\frac{\mu_i}{\mu_0} = \left(1 - \frac{[UO_2^{2+}]}{[UO_2^{2+}]_{crit}} \right)^n \quad (1)$$

where μ_i is the first-order growth rate in the presence of UO_2^{2+} , μ_0 is the growth rate in UO_2^{2+} free medium, and $[\text{UO}_2^{2+}]$ is the concentration (in μM) in the medium. The $[\text{UO}_2^{2+}]_{\text{crit}}$ value corresponds to the predicted minimum $[\text{UO}_2^{2+}]$ that completely inhibits growth based on a best fit linear regression of Eqn. 1. The exponent, n , is referred to as the “toxic power”. The quotient on the left side of Equation 1, hereafter referred to as the relative inhibition, was plotted against the UO_2^{2+} concentration to generate toxicity curves. Cell-free and carbon-free controls were performed in parallel. The $[\text{UO}_2^{2+}]_{\text{crit}}$ and n values were calculated according to Levenspiel (1980). The calculated $[\text{UO}_2^{2+}]_{\text{crit}}$ values were confirmed empirically. Experiments were performed in triplicate. Cell-free and carbon-free controls were performed in parallel.

Uranium Speciation Modeling

Uranium speciation in substrate-free media was determined using Visual MINTEQ, ver. 2.52. The partial pressure of atmospheric CO_2 was incorporated into the model and was assumed to stay constant at 38.5 Pa. Due to a lack of published thermodynamic data, PIPES buffer was not included in the modeling. Incorporation of the four substrates was also excluded, again due to a lack of published thermodynamic data for all substrates. Thermodynamic data were available for butyrate and lactate, but these substrates were not predicted to alter UO_2^{2+} speciation significantly.

Statistical Analysis

All reported values represent the mean of triplicate experiments, and error bars correspond to 95% confidence intervals. Single factor ANOVA was performed on the calculated parameters. Results were determined to be significantly different if $p < 0.05$.

Results and Discussion

UO₂²⁺ Toxicity and Modeling Results

Figure 4 shows the growth curves of isolate A on each of the four substrates over a range of [UO₂²⁺] (0 – 150 μ M) in low bicarbonate media (see Appendix A for raw data). With the exception of ethanol as a substrate, cells tolerated [UO₂²⁺] up to 50 μ M without significant inhibition, and when grown on either butyrate or dextrose, [UO₂²⁺] up to 100 μ M showed only moderate inhibition both in terms of growth rate and cell yield. Cultures grown on lactate were significantly inhibited by 100 μ M UO₂²⁺. Remarkably, cells grown on ethanol were sensitive to [UO₂²⁺] as low as 0.1 μ M, a concentration which falls below the United States Environmental Protection Agency (US-EPA) prescribed drinking water limit of 0.126 μ M. To our knowledge, this is the first reported example of sub-micromolar UO₂²⁺ concentrations inhibiting microbial growth.

Equation 1 was used to model the results obtained for each substrate, the results of which are shown graphically in Figure 5. Important parameters are summarized in Table 2. UO₂²⁺ toxicity to isolate A was dependent on the carbon source tested, as indicated by the significantly different n terms. However, the three [UO₂²⁺]_{crit} values associated with growth on butyrate, dextrose, and lactate were not found to be

statistically different ($p < 0.05$) and averaged $154 \mu\text{M}$. Growth on ethanol produced a dramatically different $[\text{UO}_2^{2+}]_{\text{crit}}$ value of $1.0 \pm 0.22 \mu\text{M}$. The empirically generated $[\text{UO}_2^{2+}]_{\text{crit}}$ values did not differ significantly from those derived using Eqn. 1, and were found to be (in μM) 160, 1.0, 150, and 145 for cells growing on butyrate, ethanol, dextrose, and lactate, respectively. The impact of the toxic power can be seen when the IC_{50} values are compared between the four substrates (Table 2). As n approaches zero, the inhibition curve becomes more sharply concave downward and the IC_{50} approaches $\text{UO}_2^{2+}_{\text{crit}}$. As n approaches 1, the inhibition curve becomes more linear, and IC_{50} approaches $\frac{1}{2} \text{UO}_2^{2+}_{\text{crit}}$. For $n > 1$, which is the case for the ethanol system, the corresponding inhibition curve becomes concave upward as IC_{50} approaches zero. For this reason, the IC_{50} values varied significantly between all four substrates. Cells grown on butyrate had the highest IC_{50} ($138 \pm 8 \mu\text{M}$) followed by dextrose ($103 \pm 3 \mu\text{M}$), lactate ($91 \pm 6 \mu\text{M}$) and ethanol ($0.19 \pm 0.04 \mu\text{M}$) (Table 2). Butyrate, which yielded the highest IC_{50} value, also yielded the lowest n value of 0.38 ± 0.10 . This trend continued with dextrose ($n = 0.62 \pm 0.09$), lactate ($n = 0.75 \pm 0.07$), and ethanol ($n = 3.3 \pm 0.20$).

In high bicarbonate media, isolate A is equally sensitive to UO_2^{2+} regardless of substrate, including ethanol (Figure 6) (see Appendix B for growth curves). A single n and $\text{UO}_2^{2+}_{\text{crit}}$ value was sufficient to characterize all the data collected under these conditions with 95% confidence (Table 2). The $\text{UO}_2^{2+}_{\text{crit}}$ value was $217 \pm 20 \mu\text{M}$, and the n value was calculated to be 0.57 ± 0.16 . Isolate A was more than two orders of magnitude more tolerant to UO_2^{2+} when grown on ethanol, due to the addition of 10 mM bicarbonate.

The significant impact of adding bicarbonate on UO_2^{2+} toxicity to isolate A can be partially explained using MINTEQA2 speciation modeling (ver. 2.52) (Table 3). These results are similar to previous studies (22,28-30), where UO_2^{2+} toxicity was mitigated by the presence of tightly binding UO_2^{2+} ligands, including bicarbonate species (22,29), and dissolved organic carbon (DOC) (28), both of which can form stable UO_2^{2+} complexes (28). Stability constants for UO_2^{2+} -bicarbonate complexes range from 8.0 to 20.0, compared to the stability constants of UO_2^{2+} -hydroxide complexes which range from -3.0 to -5.0 (31). The bioavailability of UO_2^{2+} , and thus toxicity, appears to correlate with stability of the UO_2^{2+} complexes present in solution. It is expected that UO_2^{2+} toxicity will depend heavily on the abundance of weakly bound UO_2^{2+} -hydroxide complexes which appear to more readily interact with important cellular functions.

In high bicarbonate media, MINTEQA2 predicted that UO_2^{2+} would be mostly present as $\text{UO}_2(\text{CO}_3)_3^{4-}$ (43% of total component concentration), UO_2CO_3 (35% of total component concentration), and $\text{Ca}_2\text{UO}_2(\text{CO}_3)_3$ (18% of total component concentration). The balance of the UO_2^{2+} (4% of total component concentration) was present as either phosphate complexes (UO_2HPO_4 , and UO_2PO_4^-) or minor bicarbonate complexes ($((\text{UO}_2)_2\text{CO}_3(\text{OH})_3^-$, $\text{UO}_2(\text{CO}_3)_2^{2-}$, and $\text{CaUO}_2(\text{CO}_3)_3^{2-}$) (Table 3). In low bicarbonate media, MINTEQA2 predicted that UO_2^{2+} aqueous equilibrium speciation would be dominated by $(\text{UO}_2)\text{CO}_3(\text{OH})_3^-$, accounting for 84% of the total component concentration, followed by UO_2PO_4^- (10% of total component concentration), and UO_2HPO_4 (2.5% of total component concentration). However, 2.5% of the total component concentration was predicted to be present as uranyl-hydroxide complexes,

which were not present in the high bicarbonate system. Consistent with previous studies, isolate A appears less sensitive to aqueous UO_2^{2+} tightly bound to bicarbonate and phosphate complexes than to UO_2^{2+} present as less stable hydroxide complexes. Even though a relatively small amount of the UO_2^{2+} present was weakly bound by hydroxides, the difference in associated bioavailability appears to cause the overall UO_2^{2+} toxicity to be significantly enhanced, particularly to the cells growing on ethanol.

Parallel experiments were performed to investigate the influence carbon source and added bicarbonate has on UO_2^{2+} bioaccumulation (Figure 7) (see Appendix E for raw data). Previous studies have demonstrated the ability of *Pseudomonas* spp. to rapidly accumulate significant amounts of UO_2^{2+} (18,19). Our results showed that between the filtered samples and the digested cells, virtually all the UO_2^{2+} originally added could be recovered. This suggested that a minimal amount of UO_2^{2+} was being washed off the cell surface, and that virtually all UO_2^{2+} was accumulated within the cell (see Appendix F for transmission electron microscopy images of Isolate exposed to UO_2^{2+}). Figure 7 shows the concentration of UO_2^{2+} accumulated ($\mu\text{mol UO}_2^{2+}$ per $\text{mg}\cdot\text{protein}$ basis) where, consistent with previous results (32), our results showed the type of carbon source had a significant impact on UO_2^{2+} accumulation. In low bicarbonate media, actively metabolizing cells of isolate A accumulated between 56% and 88% of the UO_2^{2+} present. Compared to the maximum UO_2^{2+} accumulation of $0.625 \mu\text{mol}/\text{mg}\cdot\text{protein}$ possible, cells metabolizing dextrose accumulated the most UO_2^{2+} ($0.56 \mu\text{mol}/\text{mg}\cdot\text{protein}$), while the lactate system accumulated the least ($0.35 \mu\text{mol}/\text{mg}\cdot\text{protein}$). Cells metabolizing butyrate and ethanol accumulated $0.44 \mu\text{mol UO}_2^{2+}/\text{mg}\cdot\text{protein}$ and $0.40 \mu\text{mol UO}_2^{2+}/\text{mg}\cdot\text{protein}$

respectively. Carbon free and heat killed controls accumulated the least amount of UO_2^{2+} (0.17 and 0.16 $\mu\text{mol UO}_2^{2+}/\text{mg}\cdot\text{protein}$ respectively). This suggests that actively metabolizing cells accumulate significantly more UO_2^{2+} , which is consistent with a previous study which used granular biomass for UO_2^{2+} removal from aqueous systems (9).

Bioaccumulation of UO_2^{2+} by isolate A (Figure 4) was significantly affected by carbon source in the high bicarbonate system as well. Among the metabolizing cells, those in the dextrose medium accumulated the most UO_2^{2+} (0.12 $\mu\text{mol UO}_2^{2+}/\text{mg}\cdot\text{protein}$), while cells metabolizing lactate accumulated the least (0.066 $\mu\text{mol UO}_2^{2+}/\text{mg}\cdot\text{protein}$). However, on average, metabolizing cells only accumulated 20% as much UO_2^{2+} as compared to cells in low bicarbonate media. The carbon free and heat killed controls accumulated the least amount of UO_2^{2+} (0.054 and 0.062 $\mu\text{mol UO}_2^{2+}/\text{mg}\cdot\text{protein}$ respectively).

The limited ability of isolate A to accumulate UO_2^{2+} in high bicarbonate media can be explained using speciation modeling results obtained by MINTEQA2 (ver. 2.52) (Table 3). In both systems, MINTEQA2 predicted that UO_2^{2+} aqueous equilibrium speciation would be dominated by neutrally or negatively charged bicarbonate and phosphate complexes. As described earlier, in low bicarbonate media, MINTEQA2 predicted that UO_2^{2+} aqueous equilibrium speciation would include uranyl-hydroxide complexes, absent in high bicarbonate media. These complexes included neutral species ($\text{UO}_2(\text{OH})_2$), negatively charged species ($\text{UO}_2(\text{OH})_3^-$), and, further unique to this system, positively charged species, including UO_2OH^+ , $(\text{UO}_2)_3(\text{OH})_5^+$, and $(\text{UO}_2)_4(\text{OH})_7^+$ (Table

2). While only predicted to be a small percentage of the total UO_2^{2+} in the system at equilibrium (2.25%), the presence of these positively charged species could explain the increased potential for bioaccumulation when the negative zeta potential of *Pseudomonas* spp. isolates is taken into consideration (33,34). Positively charged UO_2^{2+} complexes will be electrostatically attracted to the surface of the cells. This is in contrast to the high bicarbonate media, in which none of the UO_2^{2+} is predicted to be present as a positive complex and therefore not electrostatically attracted to the cells.

In addition, electrostatic considerations can explain the relatively minimal accumulation observed for heat killed and carbon limited cells. Recent experiments with *Pseudomonas aeruginosa* (ATCC 10145) showed that starved and dead cells had less negative zeta potentials compared to metabolically active cells (34). This difference may partially explain why starved and killed cells would accumulate less positively charged species. Similar reasoning explains why starved and heat killed cells accumulated relatively more UO_2^{2+} in the presence of high bicarbonate compared UO_2^{2+} accumulated in the absence of added bicarbonate (Figure 7). Heat killed and carbon limited cells accumulated $74 \pm 17\%$ and $65 \pm 26\%$ as much UO_2^{2+} as the metabolizing cells, respectively in high bicarbonate media. In the low bicarbonate media, only $37 \pm 2\%$ and $40 \pm 2\%$ as much UO_2^{2+} was accumulated by the heat killed and carbon limited cells relative to the active cells. Positively charged UO_2^{2+} species will be less electrostatically attracted to killed and starved cells, so too will negatively charged species be less electrostatically repulsed from killed and starved cells.

Electrostatic considerations do not explain the influence of carbon source on UO_2^{2+} accumulation, since in both high and low bicarbonate media cells metabolizing dextrose accumulated the most UO_2^{2+} . Substrate binding to UO_2^{2+} also does not appear to explain the observed accumulation patterns, as there is no correlation between the extent of accumulation in cells metabolizing a particular substrate, and the ability of that substrate to form stable complexes with UO_2^{2+} . Of the four substrates tested, only lactate and butyrate form complexes with UO_2^{2+} . In low carbonate media cells metabolizing these substrates accumulated the least and second most UO_2^{2+} , respectively. However, metabolism does appear to impact UO_2^{+2} bioaccumulation in isolate A.

The UO_2^{2+} speciation modeling results explained many of the toxicity and bioaccumulation patterns observed between high and low bicarbonate systems. Our results show, between the two systems, an 80% decrease in UO_2^{2+} bioaccumulation corresponded with an average 40% increase in UO_2^{2+} tolerance. However, this trend was not conserved across substrates within either the high or low bicarbonate systems. If a strong correlation between accumulation and toxicity existed, it would be expected that conditions that lead to more UO_2^{2+} accumulation would also lead to proportionately greater UO_2^{2+} toxicity. However, in low bicarbonate media, cells metabolizing ethanol accumulated only 24% less UO_2^{2+} than cells metabolizing dextrose, but the IC_{50} of cells growing on ethanol was only 0.2% that of cells growing on dextrose. In high bicarbonate media, bioaccumulation depended on the carbon sources tested, but toxicity did not, indicating that UO_2^{2+} accumulation in the presence of 10 mM bicarbonate has negligible effect on toxicity. While adding bicarbonate reduced both bioaccumulation and toxicity,

it did not do so proportionately (the five-fold decrease in accumulation led to a less than two-fold reduction in UO_2^{2+} toxicity). To our knowledge, no studies in which correlations between UO_2^{2+} toxicity and accumulation in bacterial cells have been published. Our results are in contradiction to a study of UO_2^{2+} and a *Chlorella* sp. which found that a two-fold increase in intracellular UO_2^{2+} resulted in a roughly two-fold increase in UO_2^{2+} toxicity (30). Ongoing research is aimed at understanding the relationship between UO_2^{2+} toxicity and bioaccumulation.

Conclusions

The effect of cellulose breakdown products on UO_2^{2+} toxicity and bioaccumulation within an environmental *Pseudomonas* sp. isolate (isolate A) under aerobic conditions was investigated. Among the breakdown products considered, the presence of high bicarbonate concentrations was found to have the most significant impact on both UO_2^{2+} toxicity and bioaccumulation. UO_2^{2+} was found to be more toxic in systems with low bicarbonate species concentrations, a result attributed to the predicted presence of unstable UO_2^{2+} -hydroxide complexes. This was especially true when cells were grown on ethanol. Under these conditions, cells were inhibited by UO_2^{2+} concentrations which fell below the EPA drinking water limits. In media with high bicarbonate concentrations, UO_2^{2+} was predicted to be present mostly as stable UO_2^{2+} -bicarbonate complexes, which were found to be less toxic due to their limited bioavailability. Cells in low bicarbonate media were found to yield more UO_2^{2+} bioaccumulation. This was largely attributed to electrostatic effects predicted based on

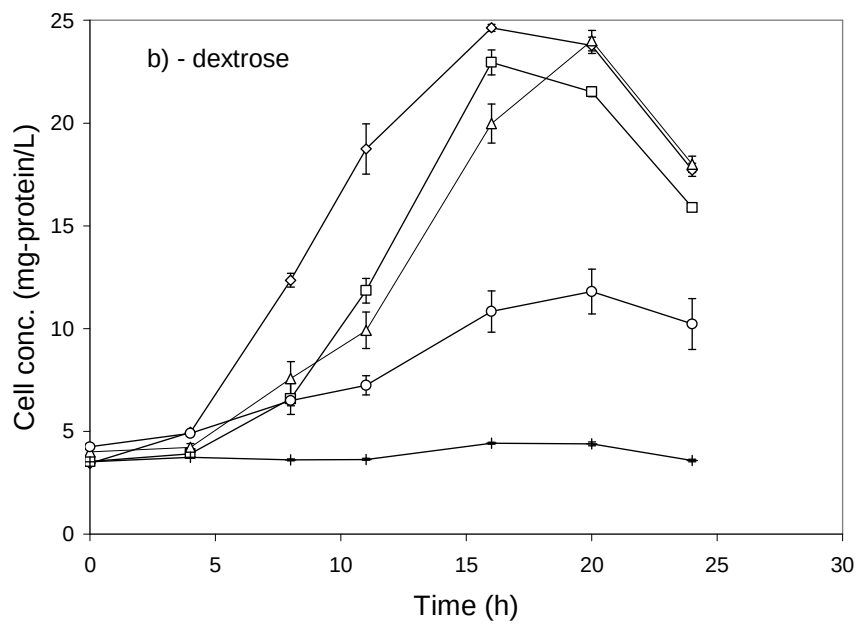
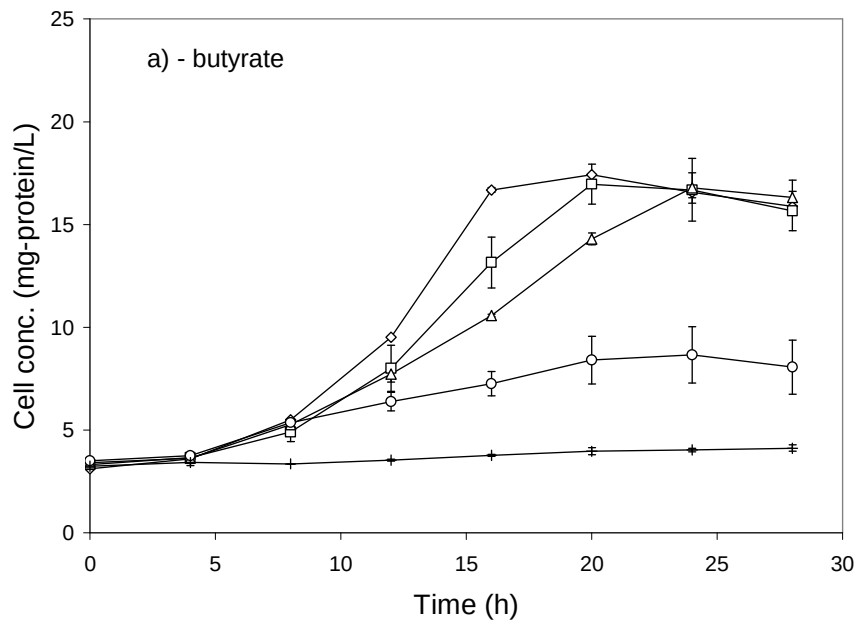
UO_2^{2+} speciation data. While UO_2^{2+} toxicity and bioaccumulation patterns could largely be explained through MINTEQ speciation modeling results, the final relationship between UO_2^{2+} toxicity and bioaccumulation remains unclear and is the subject of ongoing research.

Table 2. Summary of the kinetic parameters describing UO_2^{+2} toxicity toward isolate A in each medium combination. ANOVA was carried out on all calculated n values for both the low and high carbonate systems. Between the different substrates, the n values associated with the limited carbonate systems were found to be significantly different ($\alpha < 0.05$). Using the same α value, all n values associated with the high carbonate systems were not significantly different, and thus the average of all n values are reported.

bicarbonate	carbon source	toxic power (n)	$[\text{UO}_2^{2+}]_{\text{crit}}$ (μM)	calculated IC50 (μM)
low	butyrate	0.38 ± 0.10	158 ± 11	138 ± 8
	dextrose	0.62 ± 0.09	153 ± 5	103 ± 3
	lactate	0.75 ± 0.07	151 ± 9	91 ± 6
	ethanol	3.3 ± 0.20	1.0 ± 0.22	0.19 ± 0.04
high	all c-sources	0.57 ± 0.16	217 ± 20	152 ± 14

Table 3. MINTEQ modeling results showing the expected U(VI) species present in the carbonate limited and excess carbonate systems. “NP” denotes species not present with a cutoff concentration of 10 ppm. In the presence of excess carbonate, UO_2^{+2} is mostly present as tightly bound carbonate or phosphate complexes, which might explain the apparent lower bioavailability of UO_2^{+2} . By contrast, the carbonate limited media is predicted to contain a number of loosely bound UO_2^{+2} -hydroxide complexes, which might account for the relatively higher bioavailability of UO_2^{+2} in these systems.

	low bicarbonate	high bicarbonate
Species	% of total component concentration	% of total component concentration
UO_2OH^+	0.20	NP
$(\text{UO}_2)_3(\text{OH})_5^+$	1.4	NP
$(\text{UO}_2)_4(\text{OH})_7^+$	0.65	NP
$\text{UO}_2(\text{OH})_3^-$	0.02	NP
$\text{UO}_2(\text{OH})_2$ (aq)	0.23	NP
UO_2HPO_4 (aq)	2.4	0.03
UO_2PO_4^-	10.2	0.12
$(\text{UO}_2)_2\text{CO}_3(\text{OH})_3^-$	84	1.2
UO_2CO_3 (aq)	0.73	35
$\text{UO}_2(\text{CO}_3)_2^{2-}$	0.11	1.3
$\text{UO}_2(\text{CO}_3)_3^{4-}$	NP	43
$\text{CaUO}_2(\text{CO}_3)_3^{2-}$	NP	0.96
$\text{Ca}_2\text{UO}_2(\text{CO}_3)_3$ (aq)	NP	18



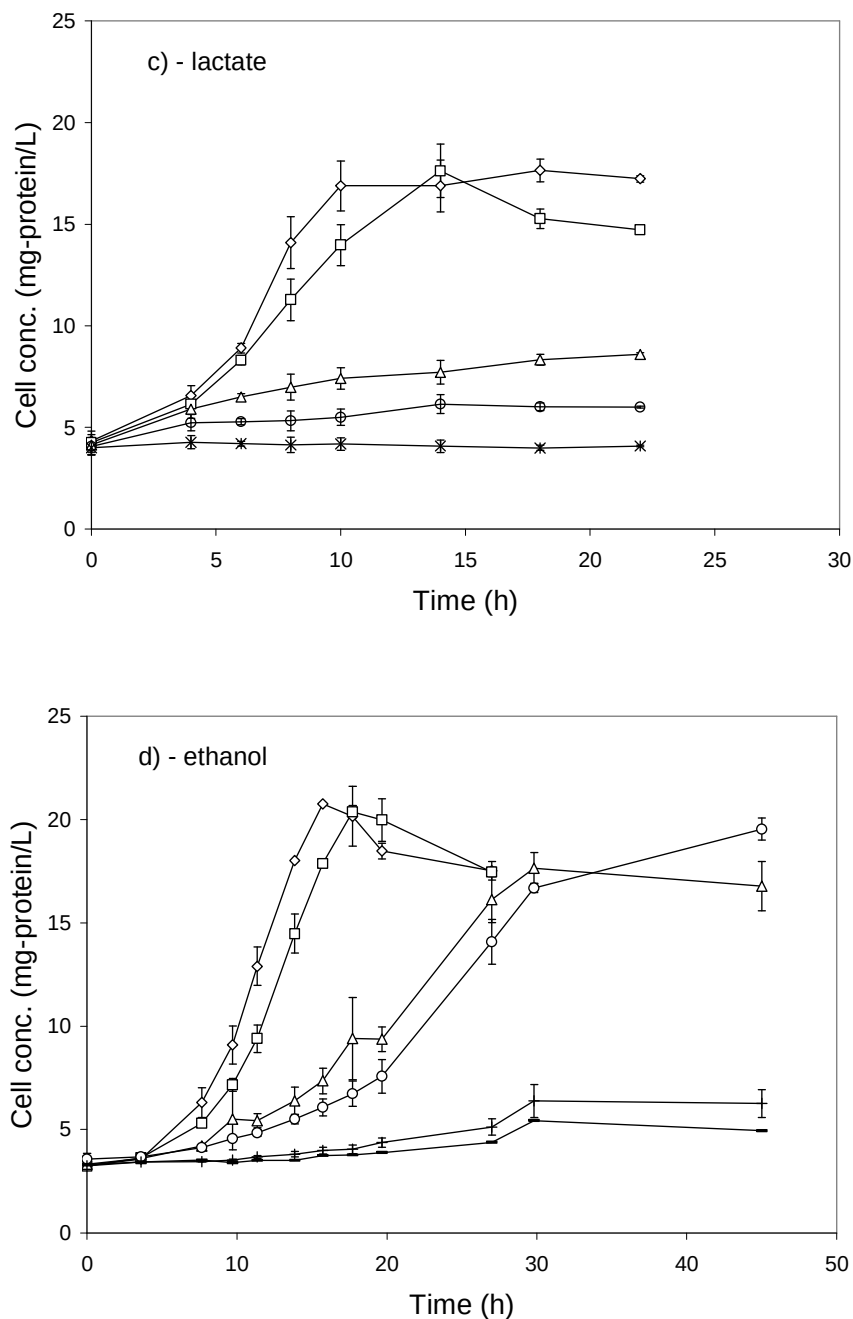


Figure 4. (a-c): Growth curves of isolate A in low carbonate media on butyrate (a), dextrose (b), and lactate (c) over a range of UO_2^{2+} concentrations ($\diamond = \text{UO}_2^{2+}$ free, $\square = 50 \mu\text{M} \text{UO}_2^{2+}$, $\Delta = 100 \mu\text{M} \text{UO}_2^{2+}$, $\circ = 125 \mu\text{M} \text{UO}_2^{2+}$, and $+$ = $150 \mu\text{M} \text{UO}_2^{2+}$). Part (d) shows growth curves of isolate A in low carbonate media on ethanol ($\diamond = \text{UO}_2^{2+}$ free, $\square = 0.10 \mu\text{M} \text{UO}_2^{2+}$, $\Delta = 0.15 \mu\text{M} \text{UO}_2^{2+}$, $\circ = 0.25 \mu\text{M} \text{UO}_2^{2+}$, $+$ = $0.50 \mu\text{M} \text{UO}_2^{2+}$, $-$ = $1.0 \mu\text{M} \text{UO}_2^{2+}$). Carbon source had a significant effect on UO_2^{2+} toxicity, particularly when ethanol served as carbon source, which led to inhibition from UO_2^{2+} concentrations as low as $0.10 \mu\text{M} \text{UO}_2^{2+}$. Error bars represent 95% confidence intervals of triplicate measurements.

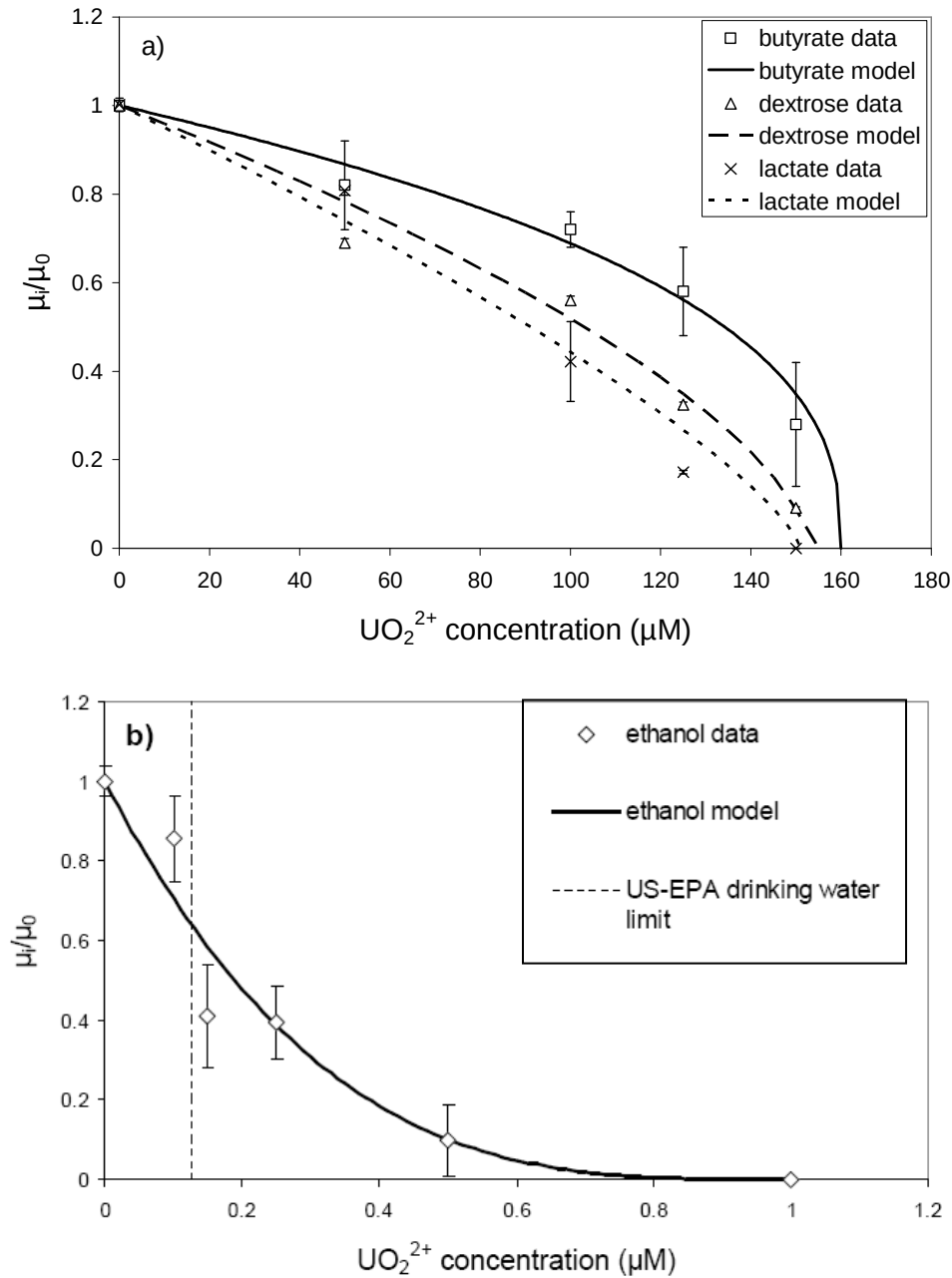


Figure 5(a-b). UO_2^{2+} toxicity modeling results: a) = butyrate, dextrose and lactate results, b) = ethanol results. Data points represent the relative inhibition induced by the corresponding UO_2^{2+} concentration. Error bars represent 95% confidence intervals of experiments performed in triplicate. Lines represent the predicted relative inhibition using Equation 1 and the kinetic parameters summarized in Table 1. UO_2^{2+} was found to be so toxic to isolate A when grown on ethanol that these modeling results had to be graphed separately. The dashed vertical line in graph b) corresponds to the US-EPA drinking water limit for U.

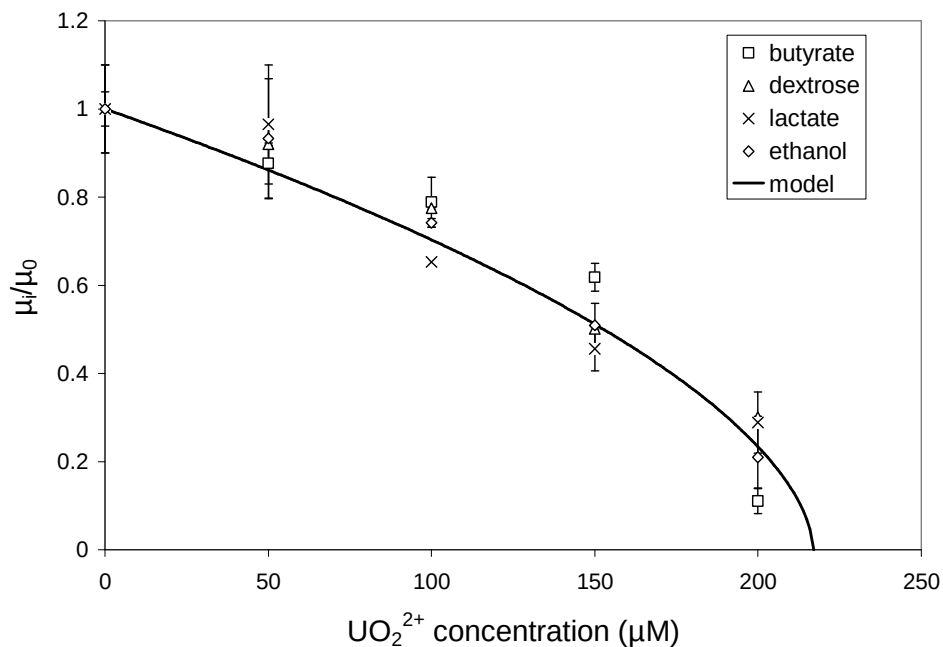


Figure 6. UO_2^{+2} toxicity and toxicity modeling results in the high carbonate systems. Data points represent the relative inhibition induced by UO_2^{+2} . Error bars represent 95% confidence intervals of experiments performed in triplicate. The line represents the predicted relative inhibition using Equation 1 and the kinetic parameters summarized in Table 2. Since the kinetic parameters did not differ significantly in the carbonate buffered systems ($p < 0.05$) only one model fit was needed to summarize the results.

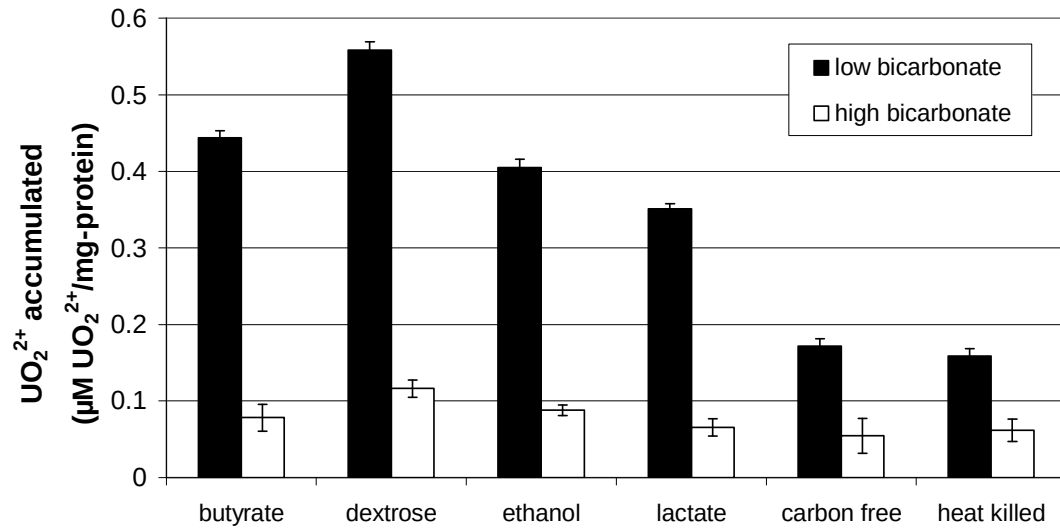


Figure 7. Micromoles UO_2^{+2} accumulated by isolate A (added to a concentration of 80 ± 5 mg-protein/L) metabolizing each of the four substrates, including carbon free and heat killed cell controls. Error bars represent 95% confidence intervals of experiments performed in triplicate.

References

1. Riley RG, Zachara JM, Wobber FJ. 1992. Chemical contaminants on DOE lands and selection of contaminant mixtures for subsurface research. DOE/ER-0547T. Department of Energy, Washington, DC.
2. Humphreys P, McGarry R, Hoffmann A, Binks P. 1997. DRINK: a biogeochemical source term model for low level radioactive waste disposal sites. *FEMS Microbiol Rev* 20:557-571.
3. Hull LC, Grossman C, Fjeld RA, Coates JT, Elzerman AW. 2004. Hybrid empirical-theoretical approach to modeling uranium adsorption. *Appl Geochem* 19:721-736.
4. Fox JR, Mortimer RJG, Lear G, Lloyd JR, Beadle I, Morris K. 2006. The biogeochemical behavior of U(VI) in the simulated near-field of a low-level radioactive waste repository. *Appl Geochem* 21:1539-1550.
5. Baston GMN, Berry JA, Bond KA, Boulton KA, Brownsword M, Linklater CM. 1994. Effects of cellulosic degradation products on uranium sorption in the geosphere. *J Alloy Compd* 213/214:475-480.
6. Bourbon X, Toulhoat P. 1996. Influence of Organic Degradation Products on the Solubilisation of Radionuclides in Intermediate and Low Level Radioactive Wastes. *Radiochim Acta* 74:315-319.
7. Moll H, Geipel G, Reich T, Bernhard G, Fanghänel T, Grenthe I. 2003. Uranyl (VI) complexes with alpha-substituted carboxylic acids in aqueous solution. *Radiochim Acta*. 91:11-20.
8. Gorman-Lewis D, Elias PE, Fein JB. 2005 Adsorption of Aqueous Uranyl Complexes onto *Bacillus subtilis* Cells. *Environ Sci Technol* 39:4906-4912.
9. Nancharaiyah YV, Joshi HM, Mohan TVK, Venugopalan VP, Narasimhan SV. 2006. Aerobic granular biomass: a novel biomaterial for efficient uranium removal. *Curr Sci* 91:503-509.
10. Selenska-Pobell S, Panak P, Miteva V, Boudakov I, Bernhard G, Nitsche H. 1999. Selective accumulation of heavy metals by three indigenous *Bacillus* strains, *B. cereus*, *B. megaterium* and *B. sphaericus*, from drain waters of a uranium waste pile. *FEMS Microbiol Ecol* 29:59-67.

11. Roden EE, Scheibe TD. 2005. Conceptual and numerical model of uranium(VI) reductive immobilization in fractured subsurface sediments. *Chemosphere* 59:617-628.
12. Sani RK, Peyton BM, Smith WA, Apel WA, Petersen JN. 2002. Dissimilatory reduction of Cr(VI), Fe(III), and U(VI) by *Cellulomonas* isolates. *Appl. Microbiol Biotechnol* 60:192-199.
13. Wall JD, Krumholz LR. 2006. Uranium Reduction. *Ann Rev Microbiol* 60:149-166.
14. Volesky B, Holan ZR. 1995. Biosorption of Heavy Metals. *Biotechnol Prog* 11:235-250.
15. Appukuttan D, Rao AS, Apte SK. 2006. Engineering of *Deinococcus radiodurans* R1 for Bioprecipitation of Uranium from Dilute Nuclear Waste. *Appl Environ Microbiol.* 72:7873-7878.
16. Powers LG, Mills HJ, Palumbo AV, Zhang C, Delaney K, Sobecky PA. 2002. Introduction of a plasmid-encoded *phoA* gene for constitutive overproduction of alkaline phosphatase in three subsurface *Pseudomonas* isolates. *FEMS Microbiol Ecol* 41:115-123.
17. Valls M, de Lorenzo V. 2002. Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. *FEMS Microbiol Rev* 26:327-338.
18. Sar P, D'Souza SF. 2001. Biosorptive uranium uptake by a *Pseudomonas* strain: characterization and equilibrium studies. *J Chem Technol Biotechnol* 76:1286-1294.
19. Strandberg GW, Shumate II, Starling E, Parrott Jr JR. 1981. Microbial Cells as Biosorbents for Heavy Metals: Accumulation of Uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa*. *Appl Environ Microbiol.* 41:237-245.
20. Francis AJ, Iden CR, Nine BJ, Chang CK. 1980. Characterization of organics in leachates from low-level radioactive-waste disposal sites. *Nuc Technol* 50:158-163.
21. Fortin C, Dutel L, Garnier-Laplace J. 2004. Uranium complexation and uptake by a green alga in relation to chemical speciation: the importance of the free uranyl ion. *Environ Toxicol Chem* 23:974-981.

22. Sani RK, Peyton BM, Dohnalkova A. 2006. Toxic Effects of Uranium on *Desulfovibrio desulfuricans* G20. *Environ Toxicol Chem* 35:1231-1238.
23. Mincher BJ, Fox RV, Cooper DC, Groenewold GS. 2003. Neptunium and plutonium sorption to Snake River Plain, Idaho soil. *Radiochim Acta* 91:397-401.
24. Atlas RM. 2004. *Handbook of microbiological media*. CRC Press, Boca Raton, FL, USA.
25. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254.
26. Hassler CS, Slaveykova VI, Wilkinson KJ. 2004. Discriminating between intra- and extracellular metals using chemical extractions. *Limnol Oceanogr-Meth* 2:237-247.
27. Levenspiel O. 1980. The Monod Equation: A Revisit and a Generalization to Product Inhibition Situations. *Biotechnol Bioeng* 22:1671-1687.
28. Hogan AC, van Dam RA, Markich S, Camilleri C. 2005. Chronic toxicity of uranium to a tropical green alga (*Chlorella* sp.) in natural waters and the influence of dissolved organic carbon. *Aquatic Toxicol* 75:343-353.
29. Riethmuller N, Markich SJ, van Dam RA, Parry D. 2001. Effects of water hardness and alkalinity on the toxicity to a tropical freshwater hydra (*Hydra viridissima*). *Biomarkers* 6:45-51.
30. Franklin NM, Stauber JL, Markich SJ, Lim RP. 2000. pH-dependent toxicity of copper and uranium to a tropical freshwater alga (*Chlorella* sp.). *Aquatic Toxicol* 48:275-289.
31. Guillaumont R, Fanghänel T, Fuger J, Grenthe I, Neck V, Palmer DA, Rand MH. 2003. *Chemical thermodynamics Volume 5: Update on the chemical thermodynamics of Uranium, Neptunium, Plutonium, Americium, and Technetium*. Elsevier B. V., Amsterdam, The Netherlands.
32. Malekzadeh F, Latifi AM, Shahamat M, Levin M, Colwell RR. 2002. Effects of selected physical and chemical parameters on uranium uptake by the bacterium *Chryseomonas* MGF-48. *World J Microb Biotechnol* 18:599-602.
33. Smets BF, Grasso D, Engwall MA, Machinist BJ. 1999. Surface physicochemical properties of *Pseudomonas fluorescens* and impact on adhesion and transport through porous media. *Colloid Surfac B: Biointerfac* 14:121-139.

34. Soni KA, Balasurbramanian AK, Beskok A, Pillai SD. 2008. Zeta potential of selected bacteria in drinking water when dead, starved, or exposed to minimal and rich culture media. *Curr Microbiol* 56:93-97.

PQQ AND QUINOPROTEINS

Introduction

Pyrroloquinoline quinone (PQQ, Figure 8) is one of five quinone cofactors and is known to be present in a variety of bacterial dehydrogenase enzymes. It is an aromatic, tricyclic *ortho*-quinone, and was the first discovered in a unique family of molecules, now recognized as the third class of redox cofactors. While much research has been reported on PQQ over the past few decades, including its synthesis, regulation, and chemistry, several questions remain, particularly those related to PQQ's overall distribution among living systems, and its redox reaction mechanisms.

Discovery and Natural Distribution of PQQ

The study of PQQ as a redox cofactor was the subject of research as early as the 1960's with the discovery of bacterial NAD(P)-independent dehydrogenases containing neither flavin nor nicotinamide (25, 3). However, the structure of PQQ was not known until Salisbury, et al. (1979) (62) and was initially given the name "methoxatin", in acknowledgment of its methylotrophic origin. It was assigned the chemical name 2,7,9-tricarboxyl-1H-pyrrolo[2,3-*f*]-quinoline-4,5-dione, but soon thereafter the name pyrroloquinoline quinone, or PQQ for short, began circulating, and PQQ-dependent enzymes became known as "quinoproteins"(14).

Since then, numerous PQQ-dependent quinoproteins have been studied, almost all of which serve as dehydrogenases in Gram-negative bacteria (16). The most well known

PQQ-dependent dehydrogenases are involved in the oxidation of alcohols and glucose (6), although PQQ-dependent sorbitol (50), glycerol (40), and formaldehyde dehydrogenases (81) have been reported. The number of processes thought to involve PQQ is still expanding, as PQQ has recently been implicated in bacterial Mn(II) oxidation (31).

Although PQQ-dependent quinoproteins were the first to be discovered, it has since been established that not all quinoproteins are PQQ-dependent (39). Four other quinoprotein cofactors have been discovered, including topaquinone (TPQ), tryptophan tryptophylquinone (TTQ), lysine tyrosylquinone (LTQ), and cysteine tryptophylquinone (CTQ). TPQ is present in copper containing amine oxidases (30), which serve a vital role in mammalian kidneys (18). TTQ is the cofactor of methylamine dehydrogenases expressed by *Methylobacterium extorquens* AM1 and *Paracoccus denitrificans* (51), and can also be found in aromatic amine dehydrogenases (24). LTQ is responsible for oxidation of lysyl residues in collagen and elastin in mammals (4). CTQ, also present in copper amine oxidases, is found in quinoprotein amine dehydrogenase in *Paracoccus denitrificans* (11). However, PQQ is unique among the quinoprotein cofactors as it is the only one not covalently bound to the enzyme. Instead, PQQ is held in place non-covalently by a Ca^{2+} ion, which also serves an important catalytic role in substrate oxidation.

Inappropriate methods of PQQ detection, established soon after its structure was elucidated, led to much confusion regarding the distribution of PQQ in living systems (15, 39, 69), and therefore reports involving PQQ, particularly those published in the

1980's, must be approached with caution. PQQ was mistakenly believed to be present as a cofactor in several eukaryotic systems, including bovine amine oxidase, fungal galactose oxidase and laccase, soybean lipoxygenase, and others (39). Many of the cofactors in these systems, wrongly identified as PQQ, were later shown to be one of the four other cofactors listed above. For example, PQQ was once believed to be the cofactor of bovine plasma amine oxidase (54) until TPQ was discovered, in part, through liquid secondary ion mass spectroscopy (LSIMS) experiments which proved PQQ is not present in bovine amine oxidase (29).

Despite past mistakes in identifying PQQ-dependent enzymes in eukaryotic systems, many have not ruled out the possible role of PQQ in higher organisms including plants and mammals, and this debate continues. PQQ has been detected in a number of food items, including tomatoes, spinach, green peppers, even beer (56), and recent evidence suggests that it is a plant growth factor (9). However, there is no evidence that plants produce PQQ. Similarly, while there is no evidence to suggest that PQQ synthesis takes place in mammals (52), many studies suggest that PQQ is an important dietary component. It has been proposed that PQQ be classified as a B vitamin, alongside niacin/nicotinic acid (vitamin B₃) and riboflavin (vitamin B₂), due to its supposed role in 2-aminoadipic 6-semialdehyde dehydrogenase (AAS) (33, 77). Supporting evidence relies heavily on murine dietary studies in which PQQ-deprived mice grew slowly, had fragile skin, showed reduced reproductive performance and neonatal growth, had compromised immune systems, and experienced altered lysine metabolism (68, 8). However, promoting PQQ to B-vitamin status has been met with resistance (19, 60), as

some have suggested that the apparent dietary benefits of PQQ intake are simply a result of its anti-oxidant properties, and not because it serves a central role in any enzymatic processes (26, 53). The role of PQQ in living systems, particularly among eukaryotic systems, therefore remains an important area of research.

PQQ Biosynthesis

PQQ is synthesized from peptide precursors containing tyrosine and glutamate residues (43). In the case of *Methylobacterium extorquens* AM1, one of the best studied systems, PQQ synthesis is regulated by *pqq* genes located in two separate clusters, *pqqABC/DE* and *pqqFG* (70). Transcription is initiated by the activation of a *pqqA* promoter by the sensor-regulatory pair MxbD and MxbM (67). PqqA is believed to be involved in the cytoplasmic synthesis of the peptide PQQ precursor, which is then acted on by the PqqEFG proteins through a series of biochemical steps not yet fully understood (73). The final step of PQQ synthesis is carried out by the PqqC/D protein through a reaction involving the cyclization and oxidation of 3a-(2-amino-2-carboxyethyl)-4,5-dioxo-4,5,6,7,8,9-hexahydroquinoline-7,9-dicarboxylic acid to form PQQ (44). This reaction requires the presence of oxygen, NAD(P)H, and a poorly understood activating protein factor (ActF) (73). The PQQ is then transported across the membrane by PqqB into the periplasm where it is incorporated into its respective enzyme (76).

PQQ and Metal Binding

Metal binding to PQQ is vitally important since all known naturally occurring PQQ-dependent quinoproteins have a Ca^{2+} bound to the PQQ. This Ca^{2+} serves both to anchor the PQQ correctly in the haloenzyme and serves a catalytic role in PQQ reduction (63, 84). Ca^{2+} binds the PQQ at the tridentate C7 carboxyl, N6, and the C5 quinone oxygen position. Other divalent metal cations, including Mg^{2+} , Sr^{2+} , and Cu^{2+} also demonstrate an affinity for this coordination environment (55, 78), and in some cases can substitute for Ca^{2+} without significant loss of enzymatic activity (46, 57). Monovalent ions, including Na^+ and K^+ , bind PQQ and other quinone cofactors at the quinone oxygens (28, 86). The ability of metals to bind PQQ at such catalytically important positions makes them potential inhibitors of PQQ-dependent enzymes. In addition, the position of most PQQ-dependent quinoproteins in the periplasm makes them particularly susceptible to environmental metal contaminants. In Chapter 4, evidence is presented which demonstrates the ability of uranium, a common soil and groundwater contaminant, to inhibit PQQ-dependent bacterial growth by binding to PQQ at the catalytic site normally occupied by Ca^{2+} .

Redox Chemistry of PQQ

PQQ is susceptible to nucleophilic attack and has a midpoint potential of +90 mV at pH 7.0 (4). The biologically relevant electron transfer reactions of PQQ take place at the C5 and C4 quinone oxygens, both of which are reduced, forming pyrroloquinoline quinol, during substrate oxidation (17). Figure 9 details this mechanism, showing

substrate oxidation, and both PQQ reduction and oxidation. Although PQQ reduction was originally thought to proceed via a nucleophilic addition-elimination (AE) reaction centered at the C5 position (20), it is now generally accepted to occur via a direct hydride transfer to C5 (85, 5, 45). However, the AE mechanism is still considered by some to be a possible mechanism of PQQ reduction in methanol dehydrogenase (MDH) (41, 27).

The hydride transfer mechanism of PQQ reduction is initiated by substrate binding and a base catalysed proton abstraction at the C1 position of the substrate (35), with the base varying between specific quinoproteins. The substrate is converted to its corresponding aldehyde, if originally an alcohol, or ketone, if a sugar. In this initial reduction step, the Ca^{2+} ion plays a catalytic role in addition to holding the PQQ and substrate in a sterically favorable position. The reduced PQQ then undergoes an enolization rearrangement before reoxidizing to PQQ via two separate single electron transfer steps (36, 37). The fate of the electrons produced during PQQ reoxidation depends on the electron transport chain components present in the specific organism under consideration, and are discussed in the following section.

PQQ Dependent Quinoproteins

Several PQQ dependent quinoproteins have been studied and described, however most of these fall within two broad categories: those that oxidize alcohols and those that oxidize glucose. They all possess a common foundational structure consisting of a propeller fold superbarrel made of eight four-stranded β -sheets, referred to as “W motifs” (71). However, they can be present as monomers, homodimers, and heterotetramers. The

following sections will discuss each class individually and provide specific examples as they appear in particular organisms.

Alcohol Oxidizing Dehydrogenases

Methanol Dehydrogenases: Methanol dehydrogenases (MDHs) oxidize methanol to formaldehyde. They are soluble, periplasmic, and have soluble *c*-type cytochromes as physiological electron acceptors (4), with the specific cytochrome depending on the organism. They are heterotetramers with $\alpha_2\beta_2$ structures (2). The α subunits are typically greater than 60 kDa in mass, and each contains one PQQ molecule (79) and one Ca^{2+} ion (22). The β subunits are much smaller (< 10 kDa), serve an unknown purpose, and do not appear in any other class of quinoproteins (6).

The MDH from *Methylobacterium extorquens* AM1 is the best studied example. Its electron transport chain proceeds from PQQ to cytochrome c_L , then to cytochrome c_H before transfer to an oxidase and finally to oxygen (2). It is basic ($\text{pI} = 8.8$), the α subunits have masses of 66 kDa, and the β subunits have masses of 8.5 kDa (42). While able to oxidize other primary alcohols, including ethanol, this MDH is unable to oxidize secondary alcohols (22). The MDH of *Paracoccus denitrificans* has been studied to a lesser extent, but it is known to have some features which differentiate it from the MDH of AM1. Its electron transport chain proceeds from PQQ to c_{551i} , then to c_{550} before transfer to a membrane bound oxidase and finally to oxygen, somewhat similar to the electron transport chain of *Pseudomonas aeruginosa* PAO1 during aerobic growth on ethanol. The α subunits have masses of 67 kDa, and the β subunits have masses of 9.5

kDa (79). Despite these differences, both enzymes have similar regulatory systems, and both are believed to be regulated by *mox* genes (22).

Alcohol Dehydrogenases: Alcohol dehydrogenases (ADH) are divided into three categories, types I, II, and III. Type I ADHs are soluble, periplasmic, and homodimeric with an α_2 configuration very similar to the α_2 units of MDH (13, 38). Unlike MDH, ADH does not have β subunits, although it was once believed they did (66). Each α subunit is ~60 kDa in size and contains one PQQ molecule. The best studied example of type I ADH is the ethanol dehydrogenase (EDH) of *Pseudomonas aeruginosa* PAO1. This enzyme is expressed during aerobic growth on ethanol (23), however it is able to oxidize other alcohols, including secondary alcohols (61). Each α subunit has been shown to contain two Ca^{2+} ions, one bound to PQQ and the other bound to the N-terminus of the enzyme (23), contrary to an earlier belief that only one Ca^{2+} was present per subunit (55). The second Ca^{2+} is believed to stabilize the conformation of the enzyme. The electron acceptor of EDH is cytochrome c_{550} , a monomeric, single-heme protein with a mass of 15 kDa (59, 64). In addition, a cytochrome bc_1 -like complex is believed to play a role in final electron transport to oxygen. The gene cluster *exaABC* regulates the expression of components necessary for ethanol oxidation. The gene *exaA* encodes the EDH haloenzyme, and *exaBC* encodes both c_{550} and a NAD^+ -dependent acetaldehyde dehydrogenase, which oxidizes acetaldehyde to acetate (65).

Unlike type I, type II and III ADHs have two prosthetic groups, including PQQ and a covalently bound heme *c* group extending from the C-terminus of the enzyme (71). Hence, these enzymes are referred to as “quinohemoproteins”. Electron transfer to the

bound heme is believed to be the first step in electron transfer from PQQ. In these enzymes, PQQ and Ca^{2+} are present in a 1:1 ratio.

Type II ADHs, like type I, are soluble and periplasmic, however they are more widely distributed than type I or III, and they are also able to oxidize a wider variety of alcohols, including vanillyl alcohol and polyvinylalcohol, in addition to ethanol (22). Type II ADHs are monomeric, have a mass ~ 70 kDa, and are found in a number of bacteria including *Pseudomonas putida* (72), *Ralstonia eutropha* (82), and *Comamonas testosteroni* (12).

Type III ADHs are quinoxemoproteins bound within the periplasmic membrane, and have only been found in acetic acid bacteria belonging to the *Acetobacter* (48), *Gluconobacter* (74), and in the recently described *Gluconacetobacter* genera (75, 21). They can contain up to three subunits, the first structurally very similar to type II ADHs. The second subunit is a tri-heme group, approximately 50 kDa in size, and the third is a smaller (~ 20 kDa) subunit not present in all type III ADHs, and whose function is not yet known. The placement of type III ADHs within the periplasmic membrane allows electrons to be directly donated to the membranous ubiquinone (UQ) pool.

Glucose Oxidizing Dehydrogenases

The known PQQ dependent glucose oxidizing dehydrogenases (GDH) can be placed into two groups: soluble GDH (sGDH) present in the periplasm, and membrane bound GDH (mGDH) located within the periplasmic membrane (47). Soluble GDH has only been found in *Acinetobacter calcoaceticus*, and is a homodimer with each subunit having a mass of 50 kDa and one PQQ molecule (58). It can catalyze the oxidation of a

number of sugars in addition to glucose, including arabinose, galactose, and a few disaccharides including lactose and maltose (22). Although sGDH has found analytical applications, for example in glucose monitors, its physiological function remains a matter of speculation.

Membrane bound GDH are more widely distributed and have been found in pseudomonads, acetic acid bacteria, and enteric bacteria (22). They are monomeric with a mass of approximately 90 kDa, and contain one PQQ molecule (10). A number of bacterial species, including *Escherichia coli*, are able to synthesize mGDH, but lack the genes for PQQ synthesis (49), and therefore PQQ must be obtained from exogenous sources (1). Unique to mGDH is its ability to substitute Mg^{2+} for Ca^{2+} at the catalytic center without any loss of activity. In fact, activity in many cases is enhanced (80).

Summary

PQQ is unquestionably an important molecule, as are the enzymes which depend on it. Understanding PQQ's interactions with potential environmental contaminants could provide valuable information related to the specific impacts such contaminants have on microbial populations. In particular, investigating this molecule and its interaction with uranium could lead to a better understanding of specific mechanisms of toxicity in bacteria. This possibility was explored, the results of which are the subject of the next chapter.

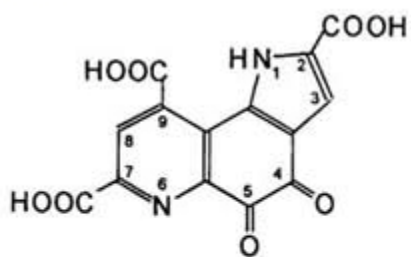


Figure 8. Structure of the pyrroloquinoline quinone (PQQ) cofactor.

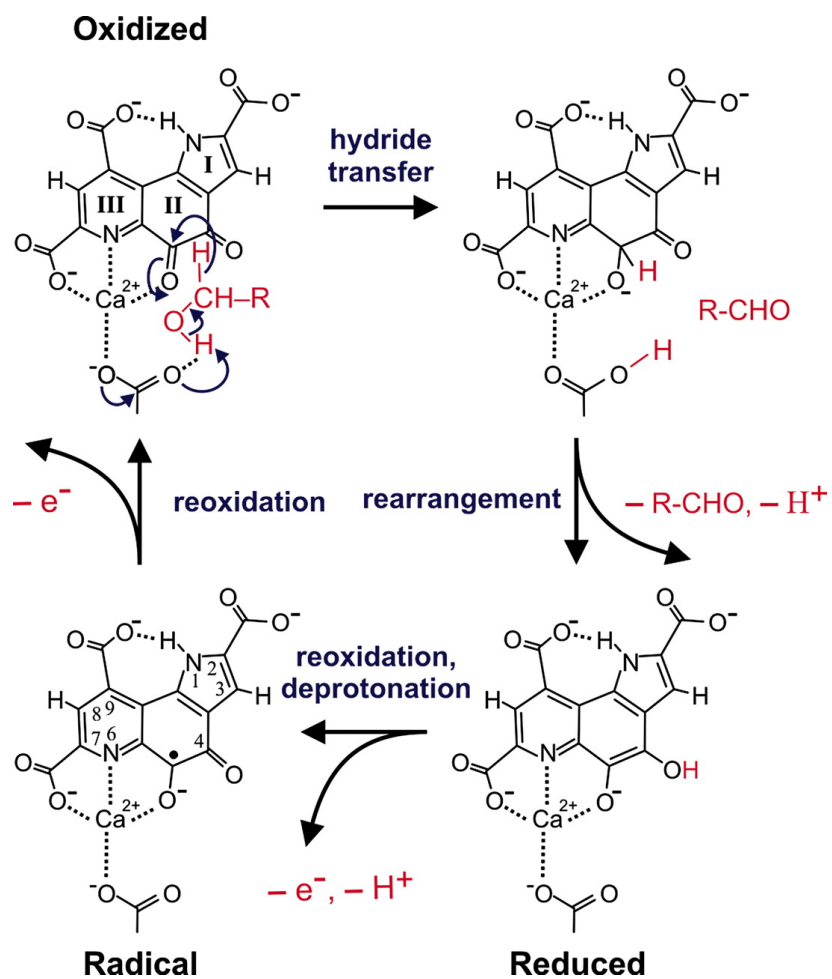


Figure 9. Hydride transfer mechanism of PQQ reduction, and mechanism of reoxidation. From Kay, et al., 2005 (35).

References

1. Adamowicz M, Conway T, Nickerson KW. 1991. Nutritional Complementation of Oxidative Glucose Metabolism in *Escherichia coli* via Pyrroloquinoline Quinone-Dependent Glucose Dehydrogenase and the Entner-Doudoroff Pathway. *Appl. Environ. Microbiol.* 57(7):2012-2015.
2. Afolabi PR, Mohammed F, Amaratunga K, Majekodunmi O, Dales SL, Gill R, Thompson D, Cooper JB, Wood SP, Goodwin PM, Anthony C. 2001. Site-Directed Mutagenesis and X-ray Crystallography of the PQQ-Containing Quinoprotein Methanol Dehydrogenase and Its Electron Acceptor, Cytochrome *c_L*. *Biochem.* 40:9799-9809.
3. Anthony C, Zatman LJ. 1967. The microbial oxidation of methanol. The prosthetic group of the alcohol dehydrogenase of *Pseudomonas* sp. M27: a new oxidoreductase prosthetic group. *Biochem. J.* 104:960-969.
4. Anthony C. 1996. Quinoprotein-catalyzed reactions. *Biochem. J.* 320:697-711.
5. Anthony C, Williams P. 2003. The structure and mechanism of methanol dehydrogenase. *Biochim. Biophys. Acta.* 1647:18-23.
6. Anthony C. 2004. The quinoprotein dehydrogenases for methanol and glucose. *Arch. Biochem. Biophys.* 428:2-9.
7. Avenzoux A, Goodwin MG, Anthony C. 1995. The role of the novel disulphide ring in the active site of the quinoprotein methanol dehydrogenase from *Methylobacterium extorquens*. *Biochem. J.* 307:735-741.
8. Bauerly KA, Storms DH, Harris CB, Hajizadeh S, Sun MY, Cheung CP, Satre MA, Fascetti AJ, Tchapanian E, Rucker RB. 2006. Pyrroloquinoline quinone nutritional status alters lysine metabolism and modulates mitochondrial DNA content in the mouse and rat. *Biochim. Biophys. Acta.* 1760:1741-1748.
9. Choi O, Jinwoo J, Kim J-G, Jeong Y, Moon JS, Park CS, Hwang I. 2008. Pyrroloquinoline Quinone Is a Plant Growth Promotion Factor Produced by *Pseudomonas fluorescens* B16. *Plant Physiol.* 146:657-668.
10. Cozier GE, Anthony C. 1995. Structure of the quinoprotein glucose dehydrogenase of *Escherichia coli* modelled on that of methanol dehydrogenase from *Methylobacterium extorquens*. *Biochem. J.* 312:679-685.

11. Datta S, Mori Y, Takagi K, Kawaguchi K, Chen Z-W, Okajima T, Kuroda S, Ikeda T, Kano K, Tanizawa K, Mathews FS. 2001. Structure of a quinohemoprotein amine dehydrogenase with an uncommon redox cofactor and highly unusual crosslinking. *Proceed. Nat. Acad. Sci.* 98(25):14268-14273.
12. de Jong GAH, Caldeira J, Sun J, Jongejan JA, de Vries S, Loehr TM, Moura I, Moura JJG, Duine JA. 1995. Characterization of the Interaction between PQQ and Heme *c* in the Quinohemoprotein Ethanol Dehydrogenase from *Comamonas testosteroni*. *Biochem.* 34:9451-9458.
13. Diehl A, Wintzingerode F, Görisch H. 1998. Quinoprotein ethanol dehydrogenase of *Pseudomonas aeruginosa* is a homodimer: Sequence of the gene and deduced structural properties of the enzyme. *Eur. J. Biochem.* 257:409-419.
14. Duine JA, Frank J, Van Zeeland JK. 1979. Glucose dehydrogenase from *Acinetobacter calcoaceticus*. *FEBS Lett.* 108(2):443-446.
15. Duine JA. 1991. Quinoproteins: enzymes cotaining the quinonoid cofactor pyrroloquinoline quinone, topaquinone, or tryptophan-tryptophylquinone. *Eur. J. Biochem.* 200:271-284.
16. Duine JA. 1999. The PQQ Story. *J. Biosci. Bioeng.* 88(3):231-236.
17. Eckert TA, Bruice TC, Gainor JA, Weinreb SM. 1982. Some electrochemical and chemical properties of methoxatin and analogous quinoquinones. *Proc. Natl. Acad. Sci.* 79:2533-2536.
18. Elmore BO, Bollinger JA, Dooley DM. 2002. Human kidney diamine oxidase: heterologous expression, purification, and characterization. *J. Biol. Inorg. Chem.* 7:565-579.
19. Felton LM, Anthony C. 2005. Role of PQQ as a mammalian enzyme cofactor? *Nature.* 433:E10.
20. Frank J, van Krimpen SH, PEJ Verwiel, JA Jongejan, Mulder AC, Duine JA. 1989. On the mechanism of inhibition of methanol dehydrogenase by cyclopropane-derived inhibitors. *Eur. J. Biochem.* 184:187-195.
21. Gómez-Manzo S, Contreras-Zentella M, González-Vlades A, Sosa-Torres M, Arreguin-Espinoza R, Escamilla-Marvá E. 2008. The PQQ-alcohol dehydrogenase of *Gluconacetobacter diazotrophicus*. *Internatl. J. Food Microbiol.* 125:71-78.

22. Goodwin PM, Anthony C. 1998. The Biochemistry, Physiology and Genetics of PQQ and PQQ-containing Enzymes. In Poole RK, Advances in Microbial Physiology, Vol 40. Academic Press, San Diego, CA, USA, pp 1-80.
23. Görisch H. 2003. The ethanol oxidation system and its regulation in *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta*. 1647:98-102.
24. Govindaraj S, Eisenstein E, Jones LH, Sander-Loehr J, Chistoserdov AY, Davidson VL, Edwards SL. 1994. Aromatic Amine Dehydrogenase, a Second Tryptophan Tryptophylquinone Enzyme. *J. Bacteriol.* 176(10):2922-2929.
25. Hauge JG. 1964. Glucose dehydrogenase of *Bacterium antiratum*: an enzyme with a novel prosthetic group. *J. Biol. Chem.* 239:3603-3639.
26. He K, Hukada H, Urakami T, Murphy MP. 2003. Antioxidant and pro-oxidant properties of pyrroloquinoline quinone (PQQ): implications for its function in biological systems. *Biochem. Pharmacol.* 65:67-74.
27. Idupulapati NB, Mainardi DS. 2008. A DMol³ study of the methanol addition-elimination oxidation mechanism by methanol dehydrogenase enzyme. *Molec. Simul.* 34(10-15):1057-1064.
28. Ishida T, Doi M, Tomita K, Hayashi H, Inoue M, Urakami T. 1989. Molecular and Crystal Structure of PQQ (Methoxatin), a Novel Coenzyme of Quinoproteins: Extensive Stacking Character and Metal Ion Interaction. *J. Am. Chem. Soc.* 111:6822-6828.
29. Janes SM, Mu D, Wemmer D, Smith AJ, Kaur S, Maltby D, Burlingame AL, Klinman JP. 1990. A new redox cofactor in eukaryotic enzymes: 6-hydroxydopa at the active site of bovine serum amine oxidase. *Science*. 248:981-987.
30. Janes SM, Palcic MM, Scaman CH, Smith AJ, Brown DE, Dooley DM, Mure M, Klinman JP. 1992. Identification of Topaquinone and Its Consensus Sequence in Copper Amine Oxidase. *Biochem.* 31:12147-12154.
31. Johnson HA, Tebo BM. 2008. In vitro studies indicate a quinone is involved in bacterial Mn(II) oxidation. *Arch. Microbiol.* 189:59-69.
32. Jongejan A, Jongejan JA, Hagen WR. 2001. Direct Hydride Transfer in the Reaction Mechanism of Quinoprotein Alcohol Dehydrogenases: A Quantum Mechanical Investigation. *J. Comput. Chem.* 22(15):1732-1749.
33. Kasahara T, Sato T. 2003. A new redox-cofactor vitamin for mammals. *Nature*. 422:832.

34. Kay CWM, Mennenga B, Görisch H, Bittl R. 2004. Characterization of the PQQ cofactor radical in quinoprotein ethanol dehydrogenase of *Pseudomonas aeruginosa* by electron paramagnetic resonance spectroscopy. *FEBS Lett.* 564:69-72.
35. Kay CWM, Mennenga B, Görisch H, Bittl R. 2005. Substrate-Binding in Quinoprotein Ethanol Dehydrogenase from *Pseudomonas aeruginosa* Studied by Electron Paramagnetic Resonance at 94 GHz. *J. Am. Chem. Soc.* 127:7974-7975.
36. Kay CWM, Mennenga B, Görisch H, Bittl R. 2006a. Structure of the Pyrroloquinoline Quinone Radical in Quinoprotein Ethanol Dehydrogenase. *J. Biol. Chem.* 281(3):1470-1476.
37. Kay CWM, Mennenga B, Görisch H, Bittl R. 2006b. Substrate binding in quinoprotein ethanol dehydrogenase from *Pseudomonas aeruginosa* studied by electron-nuclear double resonance. *Proc. Natl. Acad. Sci.* 103(14):5267-5272.
38. Keitel, T, Diehl A, Knaute T, Stezowski JJ, Höhne W, Görisch H. 2000. X-ray Structure of the Quinoprotein Ethanol dehydrogenase from *Pseudomonas aeruginosa*: Basis for Substrate Specificity. *J. Mol. Biol.* 297:961-974.
39. Klinman JP, Mu D. 1994. Quinoenzymes in biology. *Annu. Rev. Biochem.* 63:299-344.
40. Lapenaite I, Kurtinaitiene B, Razumiene J, Laurinavicius V, Marcinkeviciene L, Bachmatova I, Meskys R, Ramanavicius A. 2005. Properties and analytical application of PQQ-dependent glycerol dehydrogenase from *Gluconobacter* sp. 33. *Analyt. Chim. Acta.* 549:140-150.
41. Leopoldini M, Russo N, Toscano M. 2007. The Preferred Reaction Path for the Oxidation of Methanol by PQQ-Containing Methanol Dehydrogenase: Addition-Elimination versus Hydride-Transfer Mechanism. *Chem. Eur. J.* 13:2109-2117.
42. Liu Q, Kirchhoff JR, Faehnle CR, Viola RE, Hudson RA. 2006. A rapid method for the purification of methanol dehydrogenase from *Methylobacterium extroquens*. *Prot. Expres. Purificat.* 46:316-320.
43. Magnusson OT, Toyama H, Saeki M, Rojas A, Reed JC, Liddington RC, Klinman JP, Schwarzenbacher R. 2004a. Quinone biogenesis: Structure and mechanism of PqqC, the final catalyst in the production of pyrroloquinoline quinone. *Proc. Natl. Acad. Sci.* 101(21):7913-7918.

44. Magnusson OT, Toyama H, Saeki M, Schwarzenbacher R, Klinman JP. 2004b. The Structure of a Biosynthetic Intermediate of Pyrroloquinoline quinone (PQQ) and Elucidation of the Final Step of PQQ Biosynthesis. *J. Am. Chem. Soc.* 126:5342-5343.
45. Masgrau L, Basran J, Hothi P, Sutcliffe MJ, Scrutton NS. 2004. Hydrogen tunneling in quinoproteins. *Arch. Biochem. Biophys.* 428:41-51.
46. Matsushita K, Nonobe M, Shinagawa E, Adachi O, Ameyama M. 1987. Reconstitution of Pyrroloquinoline Quinone-Dependent D-Glucose Oxidase Respiratory Chain of *Escherichia coli* with Cytochrome *o* oxidase. *J. Bacteriol.* 169(1):205-209.
47. Matsushita K, Shinagawa E, Adachi O, Ameyama M. 1988. Quinoprotein D-glucose dehydrogenase in *Acetobacter calcoaceticus* LMD 79.41: the membrane-bound enzyme is distinct from the soluble enzyme. *FEMS Microbiol. Lett.* 55:53-58.
48. Matsushita K, Takahashi K, Takahashi M, Ameyama M, Adachi O. 1992. Methanol and Ethanol Oxidase Respiratory Chains of the Methylotrophic Acetic Acid Bacterium, *Acetobacter methanolicus*. *J. Biochem.* 111:739-747.
49. Matsushita K, Arents JC, Bader R, Yamada M, Adachi O, Postma PQ. 1997. *Escherichia coli* is unable to produce pyrroloquinoline quinone (PQQ). *Microbiol.* 143:3149-3156.
50. Matsushita K, Toyama H, Yamada M, Adachi O. 2002. Quinoproteins: structure, function, and biotechnological applications. *Appl. Microbiol. Biotechnol.* 58:13-22.
51. McIntire WS, Wemmer DE, Chistoserdov A, Lindstrom ME. 1991. A New Cofactor in a Prokaryotic Enzyme: Tryptophan Tryptophylquinone as the Redox Prosthetic Group in Methylamine Dehydrogenase. *Science.* 252:817-824.
52. McIntire WS. 1998. Newly Discovered Redox Cofactors: Possible Nutritional, Medical, and Pharmacological Relevance to Higher Animals. *Annu. Rev. Nutr.* 18:145-177.
53. Misra HS, Khairnar NP, Barik A, Priyadarsini KI, Mohan H, Apte SK. 2004. Pyrroloquinoline-quinone: a reactive oxygen species scavenger in bacteria. *FEBS Lett.* 578:26-30.

54. Moog RS, McGuirl MA, Cote CE, Dooley DM. 1986. Evidence for methoxatin (pyrroloquinolinequinone) as the cofactor in bovine plasma amine oxidase from resonance Raman spectroscopy. *Proc. Natl. Acad. Sci.* 83:8435-8439.
55. Mutzel A, Görisch H. 1991. Quinoprotein Ethanol Dehydrogenase: Preparation of the Apo-form and Reconstitution with Pyrroloquinoline Quinone and Ca^{2+} and Sr^{2+} Ions. *Agric. Biol. Chem.* 55(7):1721-1726.
56. Noji N, Hakamura T, Kitahata N, Taguchi K, Kudo T, Yoshida S, Tsujimoto M, Sugiyama T, Asami T. 2007. Simple and Sensitive Method for Pyrroloquinoline quinone (PQQ) Analysis in Various Foods Using Liquid Chromatography/Electrospray-Ionization Tandem Mass Spectrometry. *J. Agric. Food Chem.* 55:7258-7263.
57. Olsthorn AJJ, Otsuki T, Duine JA. 1997. Ca^{2+} and its substitutes have two different binding sites and roles in soluble, quinoprotein (pyrroloquinoline-quinone-containing) glucose dehydrogenase. *Eur. J. Biochem.* 247:659-665.
58. Oubrie A, Rozenboom HJ, Kalk KH, Duine JA, Dijkstra BW. 1999. The 1.7 Å Crystal Structure of the Apo Form of the Soluble Quinoprotein Glucose Dehydrogenase from *Acinetobacter calcoaceticus* Reveals a Novel Internal Conserved Sequence Repeat. *J. Mol. Biol.* 289:319-333.
59. Reichmann P, Görisch H. 1993. Cytochrome c_{550} from *Pseudomonas aeruginosa*. *Biochem. J.* 289:173-178.
60. Rucker R, Storms D, Sheets A, Tchapanian E, Fascetti, A. 2005. Is pyrroloquinoline quinone a vitamin? *Nature.* 433:E10-E11.
61. Rupp M, Görisch H. 1988. Purification, Crystallisation and Characterization of Quinoprotein Ethanol Dehydrogenase from *Pseudomonas aeruginosa*. *Biol. Chem. Hoppe-Seyler.* 369:431-439.
62. Salisbury SA, Forrest HS, Cruse WBT, Kennard O. 1979. A novel coenzyme from bacterial primary alcohol dehydrogenases. *Nature.* 280:843-844.
63. Schürer G, Clark T. 1998. Is the calcium-ion catalysis of biological reoxidation of reduced PQQ purely electrostatic? *Chem. Commun.* (2):257-258.
64. Schobert M, Görisch H. 1999. Cytochrome c_{550} is an essential component of the quinoprotein ethanol oxidation system in *Pseudomonas aeruginosa*: cloning and sequencing of the genes encoding cytochrome c_{550} and an adjacent acetaldehyde dehydrogenase. *Microbiol.* 145:471-481.

65. Schobert M, Görisch H. 2001. A soluble two-component regulatory system controls expression of quinoprotein ethanol dehydrogenase (QEDH) but not expression of cytochrome *c*₅₅₀ of the ethanol-oxidation system in *Pseudomonas aeruginosa*. *Microbiol.* 147:363-372.
66. Schrover JMJ, Frank J, Van Wielink JE, Duine JA. 1993. Quaternary structure of quinoprotein ethanol dehydrogenase from *Pseudomonas aeruginosa* and its reoxidation with a novel cytochrome *c* from this organism. *Biochem. J.* 290:123-127.
67. Springer AL, Morris CJ, Lindstrom ME. 1997. Molecular analysis of *mxbD* and *mxbM*, a putative sensor regulator pair required for oxidation of methanol in *Methylobacterium extorquens* AM1. *Microbiol.* 143:1737-1744.
68. Steinberg FM, Gershwin ME, Rucker RB. 1994. Dietary Pyrroloquinoline Quinone: Growth and Immune Response in BALB/c Mice. *J. Nutr.* 124(5):744-753.
69. Stites TE, Mitchell AE, Rucker RB. 2000. Physiological Importance of Quinoenzymes and the O-Quinone Family of Cofactors. *J. Nutrition.* 130(4):719-727.
70. Toyama H, Chistserdova L, Lindstrom ME. 1997. Sequence analysis of *pqq* genes required for biosynthesis of pyrroloquinoline quinone in *Methylobacterium extorquens* AM1 and the purification of a biosynthetic intermediate. *Microbiol.* 143:595-602.
71. Toyama H, Mathews FS, Adachi O, Matsushita K. 2004. Quinohemoprotein alcohol dehydrogenases: structure, function, and physiology. *Arch. Biochem. Biophys.* 428:10-21.
72. Toyama H, Chen Z-W, Fukumoto M, Adachi O, Matsushita K, Mathews FS. 2005. Molecular Cloning and Structural Analysis of Quinohemoprotein Alcohol Dehydrogenase ADH-IIG from *Pseudomonas putida* HK5. *J. Mol. Biol.* 352:91-104.
73. Toyama H, Nishibayashi E, Saeki M, Adachi O, Matsushita K. 2007. Factors required for the catalytic reaction of PqqC/D which produces pyrroloquinoline quinone. *Biochem. Biophys. Res. Comm.* 354:290-295.
74. Trcek J. Quick identification of acetic acid bacteria based on nucleotide sequences of the 16S-23S rDNA internal transcribed spacer region and the PQQ-dependent alcohol dehydrogenase gene. *Sys. Appl. Microbiol.* 28:735-745.

75. Trček J, Jernejc K, Matsushita K. 2007. The highly tolerant acetic acid bacterium *Gluconacetobacter europaeus* adapts to the presence of acetic acid by changes in lipid composition, morphological properties and PQQ-dependent ADH expression. *Extremophil.* 11:627-635.
76. Velterop JS, Sellink E, Meulenbergh JJM, Bulder DS, Postma PW. 1995. Synthesis of pyrroloquinoline quinone *in vivo* and detection of an intermediate in the biosynthetic pathway. *J. Bacteriol.* 177:5088-5098.
77. Wang L, Chaoneng J, Xu Y, Xu J, Dai J, Wu Q, Wu M, Zou X, Sun L, Gu S, Xie Y, Mao Y. 2005. Cloning and characterization of a novel human homolog of mouse U26, a putative PQQ-dependent AAS dehydrogenase. *Molecul. Biol. Rep.* 32:47-53.
78. Wanner M, Sixt T, Klinkhammer K-W, Kaim W. 1999. First Experimental Structure of a 1:1 Metal Complex with a PQQ Cofactor Derivative outside Dehydrogenase Enzymes. *Inorg. Chem.* 38:2753-2755.
79. Xia Z-X, Dai W-W, He Y-N, White SA, Mathews FS, Davidson VL. 2003. X-ray structure of methanol dehydrogenase from *Paracoccus denitrificans* and molecular modeling of its interactions with cytochrome *c*-551i. *J. Biol. Inorg. Chem.* 8:843-854.
80. Yamada M, Elias MD, Matsushita K, Migita CT, Adachi O. 2003. *Escherichia coli* PQQ-containing quinoprotein glucose dehydrogenase: its structure comparison with other quinoproteins. *Biochim. Biophys. Acta.* 1647:185-192.
81. Zahn JA, Bergmann DJ, Boyd JM, Kunz RC, DiSpirito AA. 2001. Membrane-Associated Quinoprotein Formaldehyde Dehydrogenase from *Methylococcus capsulatus* Bath. *J. Bacteriol.* 183(23):6832-6840.
82. Zarnt G, Schröder T, Andreessen JR. 1997. Degradation of Tetrahydrofurfuryl alcohol by *Ralstonia eutropha* Is Initiated by an Inducible Pyrroloquinoline Quinone-Dependent Alcohol Dehydrogenase. *Appl. Environ. Microbiol.* 63(12):4891-4898.
83. Zhang X, Reddy S, Bruice TC. 2007. Mechanism of methanol dehydrogenase by quinoprotein methanol dehydrogenase. *Proc. Natl. Acad. Sci.* 104(3):745-749.
84. Zheng Y-J, Bruice TC. 1997. Conformation of coenzyme pyrroloquinoline quinone and role of Ca^{2+} in the catalytic mechanism of quinoprotein methanol dehydrogenase. *Proc. Natl. Acad. Sci.* 94:11881-11886.

85. Zheng Y-J, Xia Z, Chen Z, Mathews FS, Bruice TC. 2001. Catalytic mechanism of quinoprotein methanol dehydrogenase: A theoretical and x-ray crystallographic investigation. *Proc. Natl. Acad. Sci.* 98(2):432-434.
86. Zhu Z, Davidson VL. 1998. Kinetic and chemical mechanisms for the effects of univalent cations on the spectral properties of aromatic amine dehydrogenase. *Biochem. J.* 329:175-182.

URANIUM EXERTS ACUTE TOXICITY BY BINDING TO PYRROLOQUINOLINE QUINONE

Introduction

The global distribution of uranium (U) contamination has remained a persistent environmental and human health threat for several decades. U contamination has come as a result of a number of activities including U mining (1), the production and use of depleted U for military purposes (2, 3), and in the United States from legacy wastes generated during the development of nuclear weaponry (4). The impact of bacteria and bacterial metabolism on U fate and transport has received considerable attention, particularly to the extent that microbial processes can be employed in U bioremediation efforts. These processes include reduction of hexavalent U(VI) to less soluble and less toxic U(IV) (5), and accumulation within biomass (6). Although U is known to be toxic to bacteria, no specific mechanisms of U toxicity have been reported despite the impact of U-bacterial interactions on environmental health. In this article, data are presented which demonstrate that the hexavalent dioxo-cation UO_2^{2+} can exert acute toxicity by binding to pyrroloquinoline quinone (PQQ, Figure 10), a non-covalently bound *ortho*-quinone cofactor required for a number of bacterial dehydrogenases (7). Our data show that UO_2^{2+} specifically binds at the C7 carboxyl, N6, and C5 quinone oxygen position. This site is known to be normally occupied by a Ca^{2+} ion, which serves both a catalytic role (8), and anchors the PQQ within the haloenzyme (9). Results were gathered through a combination of *in vivo*, *in vitro*, and *in silico* studies. The results presented here serve as

the first detailed mechanism of U toxicity to bacteria, and also potentially have implications for U toxicity for higher organisms, including humans.

In Vivo Studies: Inhibition of Bacteria During PQQ-Dependent Growth

Pseudomonas aeruginosa (PAO1) and *Methylobacterium extorquens* (AM1) both express periplasmic PQQ-dependent ethanol and methanol dehydrogenases, respectively, during aerobic growth on these substrates (10). For PAO1, ethanol oxidation is believed to proceed via a hydride transfer mechanism to the PQQ cofactor (11), producing reduced PQQH₂ and acetaldehyde (12). Methanol oxidation proceeds in a similar manner for AM1, producing formaldehyde (13). Growth on these specific substrates corresponded with significant UO₂²⁺ inhibition, even at sub-micromolar U concentrations (Figure 11a-d) (see Appendix G for raw data). PAO1, when growing on ethanol is significantly inhibited by 0.5 μM UO₂²⁺ (added as UO₂Cl₂), a concentration less than four times the US EPA drinking water standard (Figure 11a). This concentration extended the lag phase by 8 hours, and 1 μM UO₂²⁺ extended the lag phase by 17 hrs. With dextrose as the sole carbon source, as a control for non PQQ-dependent growth conditions, PAO1 tolerated UO₂²⁺ concentrations of 25 μM without experiencing significant inhibition, representing a more than 50-fold increase in UO₂²⁺ tolerance (Figure 11b).

This observed relationship between PQQ-dependent growth conditions and acute UO₂²⁺ toxicity was repeated with AM1 (Figure 11c-d). When grown on methanol, AM1 was significantly inhibited by 2.0 μM UO₂²⁺. Growth on methylamine led to a significant increase in UO₂²⁺ tolerance, as only moderate inhibition was observed at UO₂²⁺

concentrations of 50 μM . During growth on methylamine, AM1 expresses a periplasmic, quinoprotein with tryptophan tryptophylquinone (TTQ) as the cofactor (14). Unlike PQQ, TTQ is covalently bound to the haloenzyme, does not require a Ca^{2+} ion to function, and does not appear to correspond with acute UO_2^{2+} toxicity.

The relationship between PQQ-dependent growth conditions and acute UO_2^{2+} toxicity shown here is in agreement with a recent study with an environmental *Pseudomonas* sp. isolated from the Idaho National Laboratory (INL) (Idaho Falls, ID, USA) (15). This isolate was inhibited by sub- μM UO_2^{2+} concentrations during aerobic growth on ethanol. Aerobic growth on dextrose, lactate, or butyrate decreased UO_2^{2+} sensitivity by more than two orders of magnitude. It has since been hypothesized that the acute UO_2^{2+} toxicity shown here was related to PQQ-dependent growth, and that this dependence was a result of UO_2^{2+} binding directly to PQQ.

In Vitro Studies: Direct Evidence of UO_2^{2+} - PQQ Binding

The ability of UO_2^{2+} to bind to PQQ was studied directly using UV-Vis spectroscopy and electrospray ionization mass spectroscopy (ESI-MS). UV-Vis spectra of PQQ and its complexes with Ca^{2+} and UO_2^{2+} are shown in Figure 12 (see Appendix H for raw data). The spectrum of a 1.0 mM PQQ solution contained a broad peak centered at approximately 460 nm. The addition of an equimolar amount of Ca^{2+} (as CaCl_2) red shifted this peak slightly, a result of the interaction between the Ca^{2+} and the C5 quinone oxygen (16). However, as shown in Figure 12a, the addition of an equimolar amount of UO_2^{2+} altered the spectrum dramatically. The peak centered at 460 nm disappeared, and

absorbance at shorter wavelengths increased dramatically. Visually, the addition of UO_2^{2+} caused the PQQ solution to turn from a brick red color to a brilliant yellow. A similar spectrum was produced when UO_2^{2+} was added to a mixture of PQQ and Ca^{2+} , suggesting that UO_2^{2+} can bind to PQQ to the exclusion of Ca^{2+} . A 1 mM UO_2^{2+} solution produced a spectrum almost indistinguishable from an H_2O blank sample. The spectral changes observed here are consistent with changes recently reported by Dimitrijevic (et al., 2006) as a result of PQQ binding to coordinatively unsaturated Ti(IV) species on the surface of nano-crystalline TiO_2 particles (17). Infrared spectroscopy provided further evidence suggesting that the Ti(IV) was binding the PQQ at the C7 carboxyl, N6, and C5 quinone oxygen position, similar to Ca^{2+} in functioning enzyme.

The nature of the PQQ-UO_2^{2+} complex was investigated further using ESI-MS in negative mode (Figure 12b). A spectrum of a 10 μM PQQ solution in water and a summary of the peak assignments (labeled A-I with increasing m/z values) are shown in Figure 12b (for additional spectra, see Appendix I). The largest peak, at $m/z = 329$, corresponds to the deprotonated PQQ molecule. Consistent with previous results, the PQQ fragmented through the loss of one or more of the C2, C7, and C9 carboxyl groups as CO_2 (18). Since PQQ was added as the disodium salt, sodium adducts also appear in the spectra (at $m/z = 263, 307, 351, 373, 395$). The fragment corresponding to the peak at $m/z = 169$ could not be identified. The nine peaks produced by PQQ provided a fingerprint which was compared to spectra generated by PQQ complexes with either Ca^{2+} or UO_2^{2+} .

An equimolar mixture of PQQ and Ca^{2+} produced a spectrum with five peaks in common with the PQQ only spectrum, including those designated B, C, G, H, and I. However, the three peaks at $m/z = 323, 345, \text{ and } 367$ were unique to the PQQ + Ca^{2+} spectrum, and correspond to PQQ- Ca^{2+} complexes formed between Ca^{2+} and fragments D, E, and F, respectively.

Adding UO_2^{2+} produced a spectrum with three peaks in common with the PQQ only and PQQ + Ca^{2+} spectra, including G, H, and I. However, the remaining six peaks were unique to the PQQ + UO_2^{2+} system, and had the same spacing pattern as the PQQ spectrum. The six peaks were shifted up 330 m/z units from their corresponding peaks in the PQQ only spectrum. This suggested that UO_2^{2+} was binding to PQQ, and specifically that UO_2^{2+} was binding as the neutrally charged UO_2CO_3 complex, which has a mass of 330. Potential binding sites included any of the three carboxyl groups, as UO_2^{2+} is known to have an affinity for such a coordination environment (19), the quinone oxygens, and the Ca^{2+} binding site. In addition to providing insight into the speciation of the bound UO_2^{2+} , the PQQ + UO_2^{2+} spectra also narrows the possible UO_2^{2+} binding sites to two. The peak at $m/z = 527$ of the PQQ + UO_2^{2+} spectra resulted from UO_2CO_3 binding to fragment A, which lacks any carboxyl groups and suggests that none of the carboxyl groups on the PQQ molecule are the preferred binding site.

Additional evidence supporting UO_2^{2+} binding at the Ca^{2+} binding site was obtained from a spectrum of a mixture of 10 μM each of PQQ, Ca^{2+} , and UO_2^{2+} . While five peaks corresponding to PQQ + UO_2CO_3 complexes appeared in this spectrum, none corresponding to PQQ + Ca^{2+} complexes appeared, suggesting that UO_2^{2+} was binding

preferentially over Ca^{2+} , and that therefore both metals coordinate with PQQ at the same site. This also suggests UO_2^{2+} is able to form a stable complex with only the N6 and C5 quinone oxygen of PQQ, since the UO_2CO_3 remains bound after the loss of all carboxyl groups, including the C7 carboxyl normally involved in Ca^{2+} binding.

A number of metals are known to bind to *ortho*-quinone groups (like the one on PQQ) (20), and further evidence was needed to support our UO_2^{2+} binding site hypothesis. Evidence against UO_2^{2+} binding to the quinone oxygens is provided by the relative insensitivity of AM1 to UO_2^{2+} toxicity when growing on methylamine. Growth on this substrate requires the cofactor TTQ, another periplasmic *ortho*-quinone (14). If UO_2^{2+} inhibited AM1 during growth on methylamine by binding to the *o*-quinone oxygens of PQQ, then similar inhibition would have been expected during growth on methylamine.

In Silico Studies: Molecular Modeling of the UO_2^{2+} - PQQ System

In order to further substantiate the preference of uranyl binding to the [O...N...O] motif in positions 5,6,7 in PQQ, a series of *in silico* modeling studies was completed using density functional theory (BP86) (21, 22) with relativistically corrected effective core potentials (LANL2DZ) (23, 24) for all atoms. In addition to gas phase calculations, simulations in a polarizable solvent environment (25, 26) were carried out with water solvent parameters (dielectric constant of 80, solvent radius 1.38 Å) as implemented in Gaussian 03 (27). In addition to the careful selection of the level of theory and model

chemistry, the effect of the protonation state of PQQ and coordination environment of the uranyl cation was systematically evaluated.

Table 4 summarizes the calculated thermodynamic parameters and Figure 4 shows representative optimized molecular structures. Only the calculations carried out for the worst-case scenario with respect to coordination are reported. The fully protonated PQQ ligand has the least affinity for the uranyl cation and the protonated carboxyl in position 7 is the most comparable to the carbonyl group in position 5. With a carboxylate group in position 7, the binding of cation will be more favored as seen in the crystal structures in Figure 13 and also from simple electrostatic considerations. With the implicit solvation effects all models and simulations found considerable preference of uranyl binding to the [O...N...O] motif than to the [O...O] motif of PQQ. The [O...N...O] coordination of the naked UO_2^{2+} (Figure 13a) is about 22 kJ/mol preferred over the [O...O] coordination. This preference is reduced to 15 kJ/mol when the explicit solvent water molecules are considered (Figure 13b). We also evaluated a more realistic model relevant to the above *in vitro* studies, when the two positive charges of the uranyl cation were neutralized by a coordinated bicarbonate ligand in addition to explicit solvent water molecules completing the coordination environment. The $\text{UO}_2(\text{CO}_3)(\text{H}_2\text{O})_2$ neutral moiety can be considered as the conclusive test for the two coordination modes since in this model the advantage of ionic interaction of the uranyl cation and the carboxylate group is diminished. While the relative energy decreased considerably, the 7 kJ/mol preference in a solvated computational model can still be considered as conclusive and indicates the importance of U-N covalent interactions.

Discussion

The mechanism of UO_2^{2+} toxicity to AM1 and PAO1 under PQQ-dependent growth conditions proposed here can readily be understood using the biotic ligand model (BLM) of acute metal toxicity (28, 29). The basic assumption of the BLM is that acute metal toxicity results from the complexation of the metal with physiologically active binding sites, to the exclusion of competing ions. The toxicity of a given metal is therefore a function of the importance of the specific physiological target, the stability of the metal-ligand complex relative to complexes formed by competing ions, the concentration of the metal relative to competing ions, and the abundance of competing ligands capable of sequestering the metal. These factors were therefore maximized for UO_2^{2+} toxicity in PAO1 and AM1 under the experimental growth conditions used, as any change to the growth conditions in ways relevant to the BLM produced dramatically different results. Specifically, changing the physiological target by growing cells on PQQ-independent conditions resulted in significant mitigation of UO_2^{2+} toxicity, as seen in Figure 1, as did the addition of excess Ca^{2+} (a competing ion) or excess carbonate (a competing UO_2^{2+} -binding ligand) to the growth media (see Appendices K and L).

These results might also explain the inhibition of bacterial manganese(II) oxidation by low UO_2^{2+} concentrations. Webb et al., (2006) reported inhibition of Mn(II) oxidation by *Bacillus* sp. strain SG-1 in the presence of only 4 μM UO_2^{2+} (30). Interestingly, it has recently been suggested that PQQ is involved with bacterial Mn(II) oxidation, based on *in vitro* studies with the Mn(II) oxidizing marine organism *Erythrobacter* sp. strain SD21 (31). Biogenic manganese oxides have been shown to have

high heavy-metal binding capacities (32) and are therefore believed to play an important role in UO_2^{2+} mobility, particularly in oxic groundwater environments where UO_2^{2+} adsorption is controlled by adsorption onto mineral surfaces (33). Biogenic, manganese-based permeable reactive barriers have been suggested in UO_2^{2+} bioremediation schemes (34), however, the ability of UO_2^{2+} to directly inhibit biogenic Mn-oxide formation may have serious implications for such attempts.

Although the role of PQQ in non-bacterial organisms is still debated (35, 36), positively identifying the UO_2^{2+} binding site on PQQ could potentially have strong implications for understanding UO_2^{2+} toxicity in other organisms, including humans. In particular, the ability of UO_2^{2+} to form a stable complex with the N6 and C5 quinone oxygen of carboxyl-free PQQ may make UO_2^{2+} a potential inhibitor of flavoproteins. Flavoproteins have at their catalytic center a tri-cyclic alloxazine group, which offer a very similar coordination environment as the UO_2^{2+} binding site of PQQ. A previous report claimed UO_2^{2+} can exert “remarkable” inhibition of flavoprotein monoamineoxidase (MAO) from renal rat kidney (37). This remarkable inhibition could possibly be the result of UO_2^{2+} binding to the MAO in a manner analogous to UO_2^{2+} binding in PQQ-dependent ethanol and methanol dehydrogenases. Investigating interactions between UO_2^{2+} and flavoprotein-related molecules could therefore provide valuable, widely applicable information regarding UO_2^{2+} toxicity.

Methods Summary

Growth media composition was as described elsewhere (15). Cells were grown aerobically at 20°C in 125 mL serum bottles sealed with butyl stoppers and crimped with Al seals. Carbon sources were added to a concentration of 25 mM-carbon. Experiments were performed in triplicate, data points correspond to the average of triplicate measurements, and reported errors correspond to 95% confidence intervals. Absorbance data and spectra was measured using a Thermo Electron Multiskan Spectrum UV-Vis spectrophotometer. Mass spectra were collected using an electrospray-ionization mass spectrometer (Agilent Ion Trap 6300, model G2440DA). All mixtures were added to a concentration of 10 μ M. PQQ was added as the disodium salt (Fisher Scientific, Fair Lawn, NJ). UO₂²⁺ was added as the Cl salt (International Bio-Analytical Industries Inc., Boca Raton, Florida), as was Ca²⁺ (Fisher Scientific, Fair Lawn, NJ). Samples were prepared in nano-pure water (17.5 Ω), and were injected at a flow rate of 0.3 mL/min. Nitrogen served as the carrier gas at a flow rate of 8.0 L/min. All spectra were obtained in negative mode, and the ion spray temperature and pressure were 365°C and 75 psi, respectively.

Table 4: PQQ binding energies (kJ/mol) and entropies (J/mol K) calculated at BP86/LANL2DZ level using water polarizable continuum model.

	ΔE^a	ΔE_0^b	ΔH	ΔS	ΔG
$[\text{UO}_2]^{2+} + \text{PQQ} \rightarrow [\text{UO}_2(\text{PQQ})]^{2+}$					
[O...N...O]	6	13	11	-203	71
tautomer					
[O...O]	24	31	29	-179	83
$[\text{UO}_2(\text{H}_2\text{O})_6]^{2+} + \text{PQQ} \rightarrow [\text{UO}_2(\text{H}_2\text{O})_4(\text{PQQ})]^{2+} + 2 \text{H}_2\text{O}$					
[O...N...O]	71	53	67	245	-6
tautomer					
[O...O]	92	66	75	212	12
$[\text{UO}_2(\text{CO}_3)(\text{H}_2\text{O})_4] + \text{PQQ} \rightarrow [\text{UO}_2(\text{CO}_3)(\text{H}_2\text{O})_2(\text{PQQ})] + 2 \text{H}_2\text{O}$					
[O...N...O]	67	53	59	73	37
tautomer					
[O...O]	94	80	86	79	63

^a sum of nucleus/nucleus, electron/nucleus, and electron/electron interactions;

^b zero-point energy corrected electronic energy

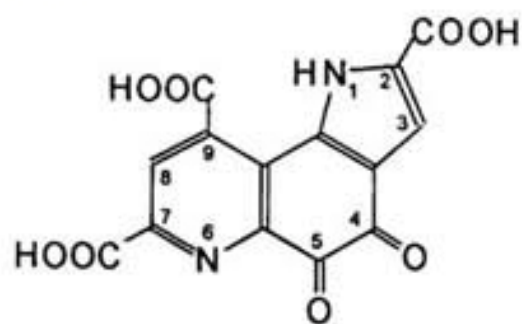


Figure 10. Structure of the pyrroloquinoline quinone (PQQ) cofactor.

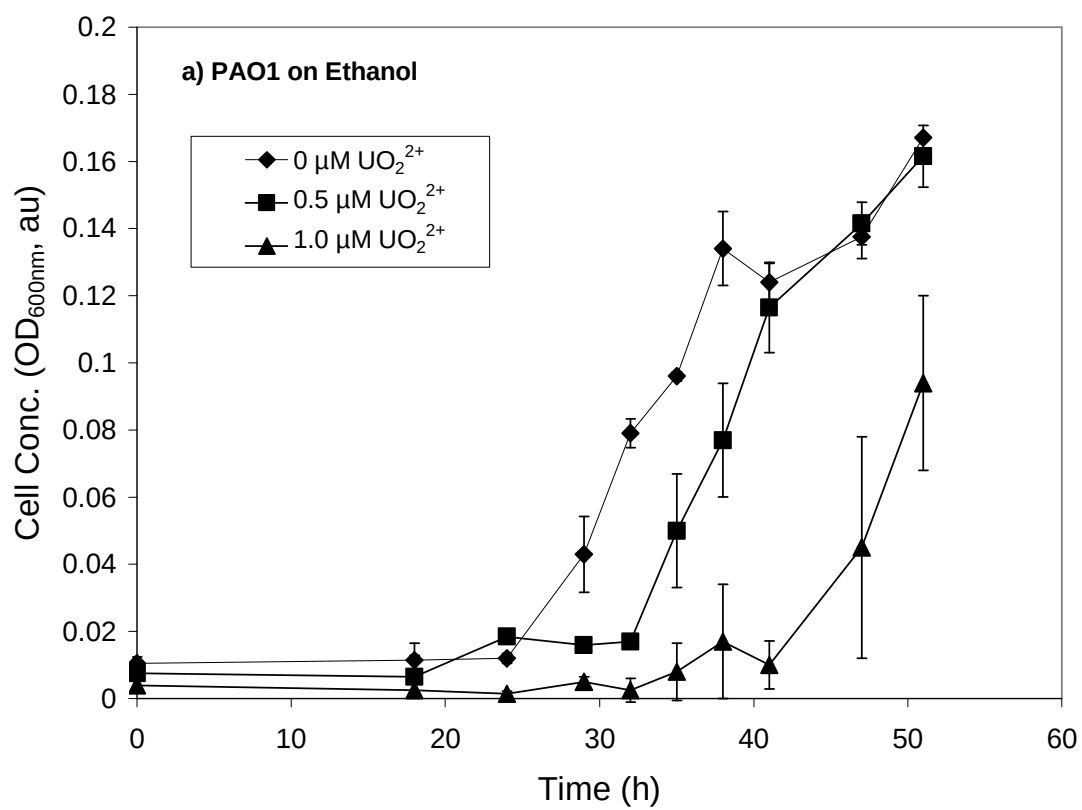


Figure 11a.

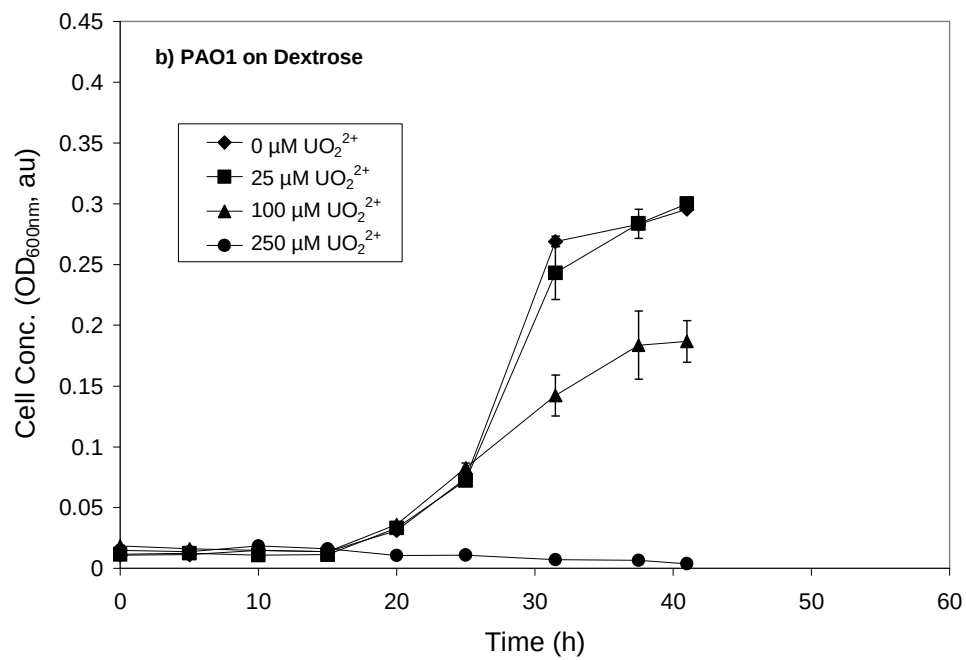


Figure 11b.

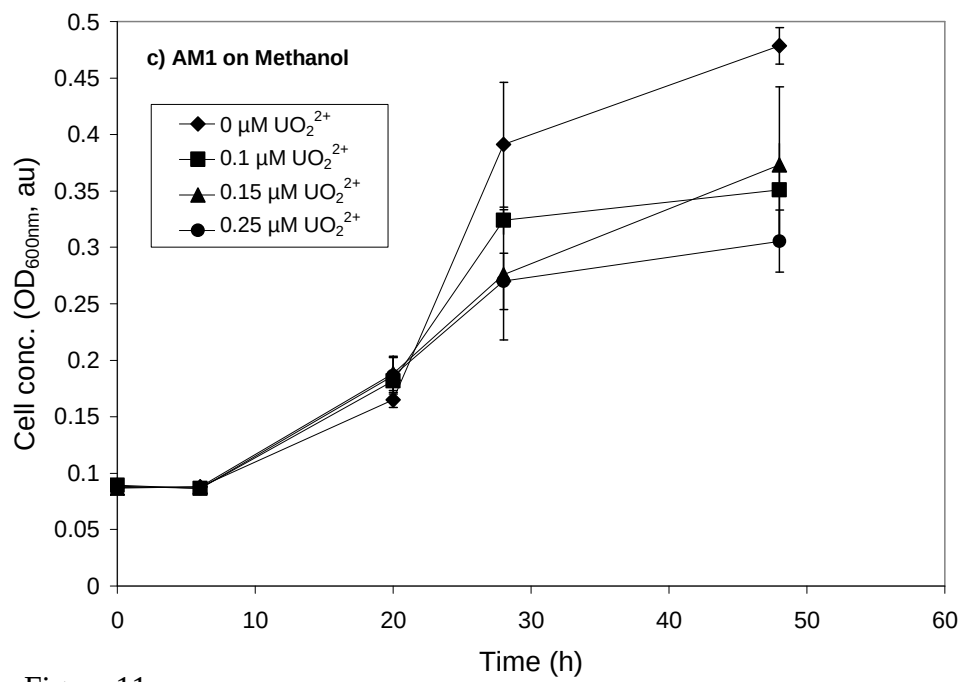


Figure 11c.

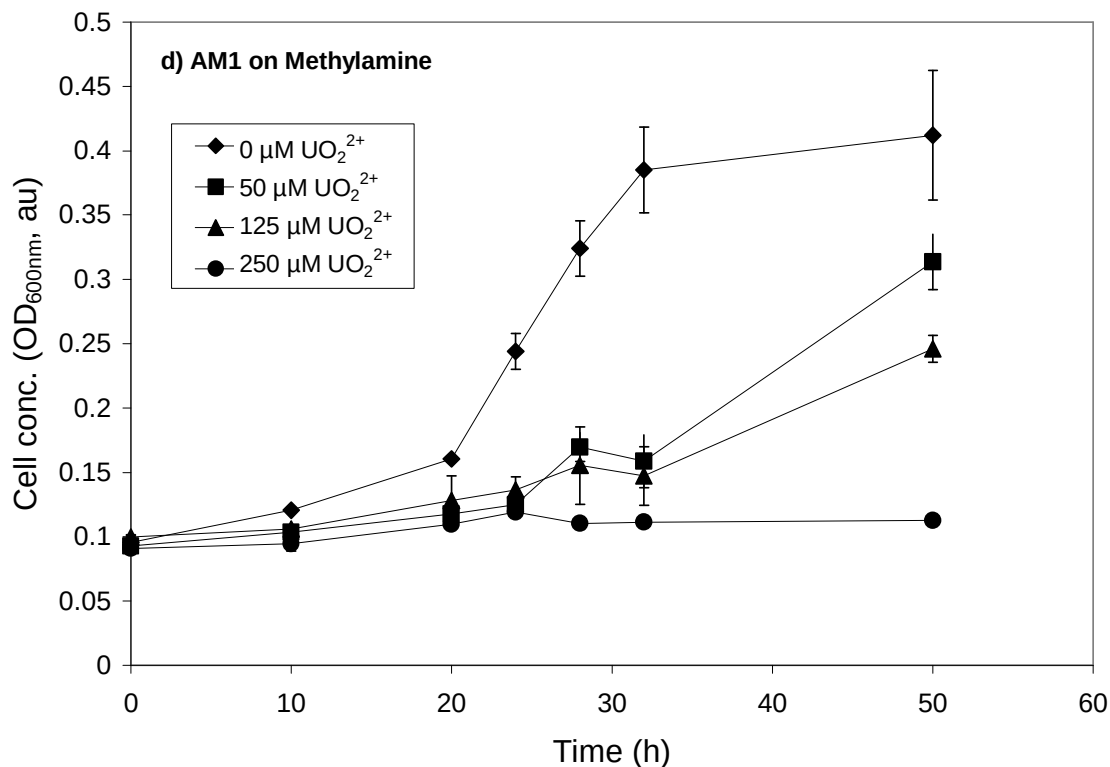


Figure 11 (a-d). Growth of PAO1 on ethanol (a) and dextrose (b) in the presence of UO_2^{2+} . Cells growing on ethanol were significantly inhibited by UO_2^{2+} concentrations as low as $0.5 \mu\text{M}$, whereas cells growing on dextrose could tolerate $25 \mu\text{M}$ UO_2^{2+} without significant inhibition. A similar pattern was seen when AM1 was grown on either methanol (c) or methylamine (d) in the presence of UO_2^{2+} . Cells growing on methanol were much more sensitive to UO_2^{2+} relative to cells growing on methylamine. This acute UO_2^{2+} toxicity is directly associated with PQQ-dependent growth conditions, suggesting that PQQ is the site of UO_2^{2+} inhibition.

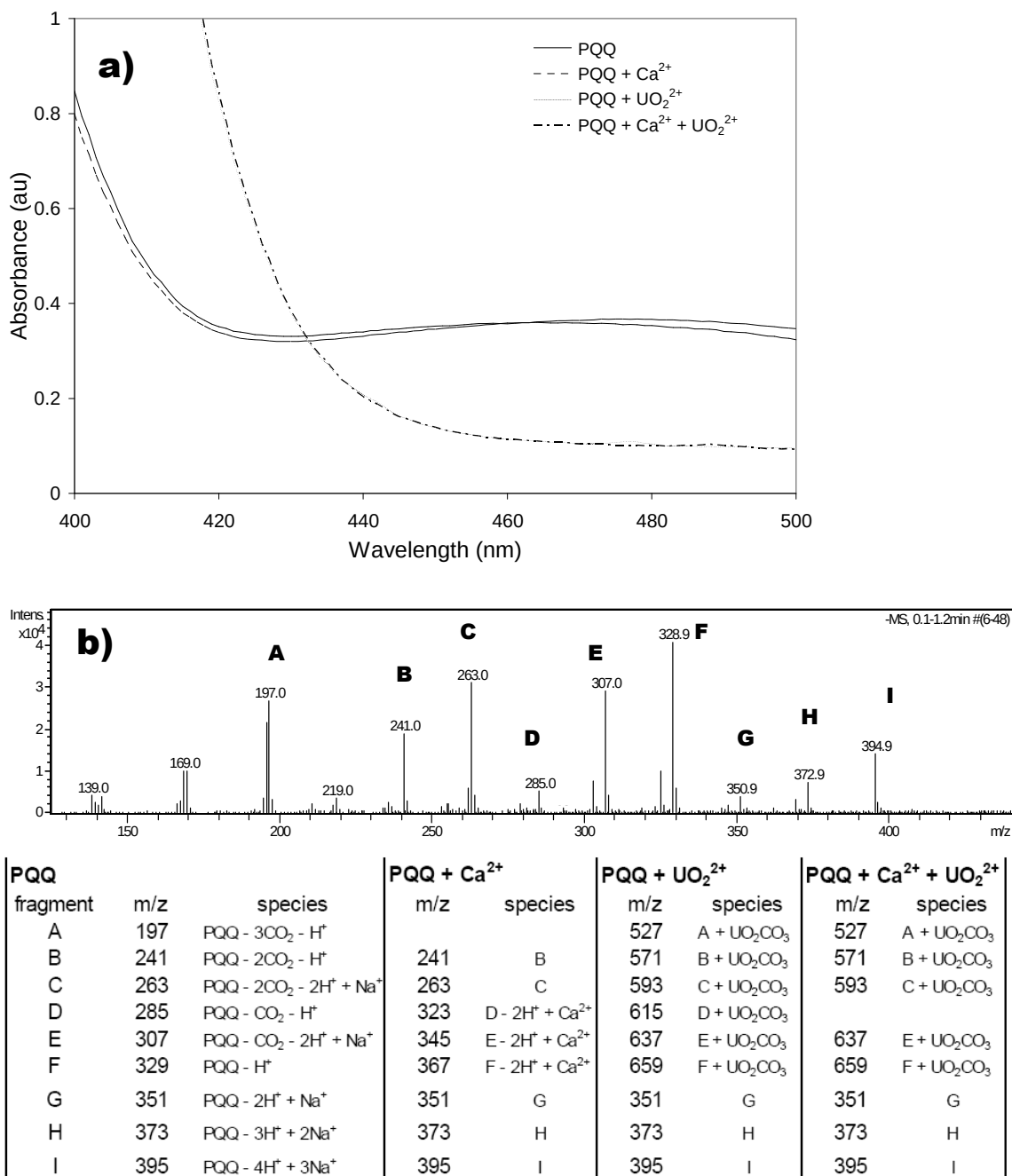


Figure 12. Results of *in vitro* studies with PQQ and UO₂²⁺. (a): UV-Vis absorbance spectra of PQQ and its complexes with Ca²⁺ and UO₂²⁺. The addition of UO₂²⁺ dramatically altered the absorbance spectrum of PQQ, suggesting that UO₂²⁺ is able to form a stable complex with PQQ. (b): Mass spectrometry of PQQ and PQQ complexes with Ca²⁺ and UO₂²⁺. The shown spectrum is of PQQ only, and prominent peaks have been labeled A – I. The left column contains the m/z value and speciation of each fragment in the PQQ only system. The three right columns summarize the peaks obtained when PQQ is equilibrated with Ca²⁺, UO₂²⁺, or both metals.

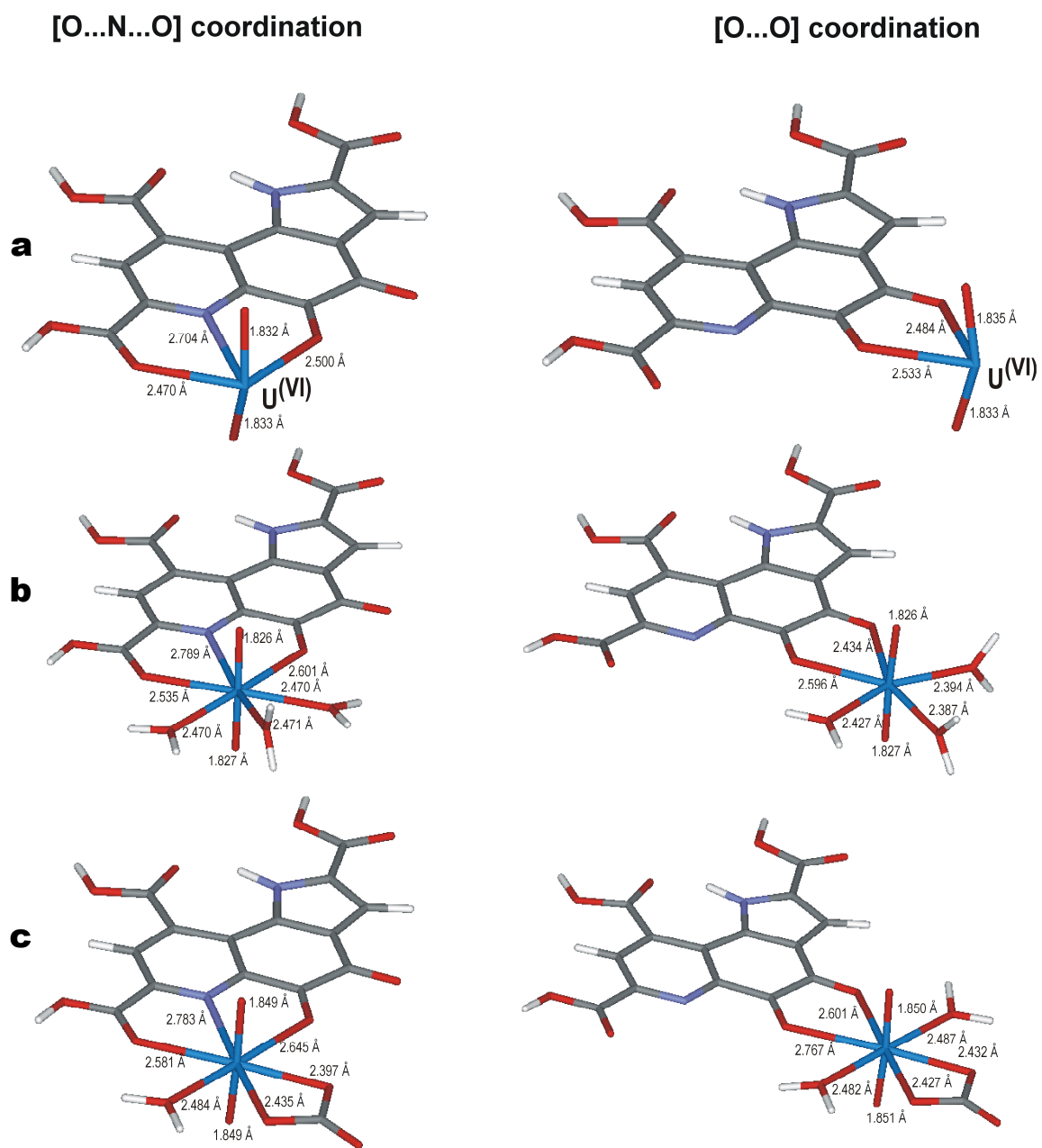


Figure 13(a-c): Optimized molecular structures with selected bond lengths for a) $[\text{UO}_2(\text{PQQ})]^{2+}$; b) $[\text{UO}_2(\text{H}_2\text{O})_4(\text{PQQ})]^{2+}$, c) $[\text{UO}_2(\text{CO}_3)(\text{H}_2\text{O})_4(\text{PQQ})]$ complexes

References

1. Landa, E. R. & Gray J. R. US Geological Survey research on the environmental fate of uranium mining and milling wastes. *Environ. Geol.* 26, 19-31 (1995).
2. Parrish, R. R. *et al.* Depleted uranium contamination by inhalation exposure and its detection after ~20 years: Implications for human health assessment. *Sci. Tot. Environ.* 390, 58-68 (2008).
3. Milačić, S., Petrović, D., Jovičić, D., Kovačević, R. & Simić, J. Examination of the health status of populations from depleted-uranium-contaminated regions. *Environ. Res.* 95, 2-10 (2004).
4. Riley, R. G. & Zachara, J. Chemical contaminants on DOE lands and selection of contaminant mixtures for subsurface research. DOE/ER-0547T. U. S. DOE (1992).
5. Wall, J.D. & Krumholz, L.R. Uranium Reduction. *Ann. Rev. Microbiol.* 60, 149-166 (2006).
6. Appukuttan, D., Rao, A. S. & Apte, S.K. Engineering of *Deinococcus radiodurans* R1 for Bioprecipitation of Uranium from Dilute Nuclear Waste. *Appl. Environ. Microbiol.* 72(12), 7873-7878 (2006).
7. Matsushita, K., Toyama, H., Yamada, M. & Adachi, O. Quinoproteins: structure, function, and biotechnological applications. *Appl. Microbiol. Biotechnol.* 58, 13-22 (2002).
8. Zheng, Y-J. & Bruce, T.C. Conformation of coenzyme pyrroloquinoline quinone and role of Ca^{2+} in the catalytic mechanism of quinoprotein methanol dehydrogenase. *Proc. Natl. Acad. Sci.* 94, 11881-11886 (1997).
9. Schürer, G. & Clark, T. Is the calcium-ion catalysis of biological reoxidation of reduced PQQ purely electrostatic? *Chem. Commun.* (2), 257-258 (1998).
10. Goodwin, P. M. & Anthony, C. The Biochemistry, Physiology and Genetics of PQQ and PQQ-containing Enzymes. In Poole RK, *Advances in Microbial Physiology*, Vol 40. Academic Press, San Diego, CA, USA, pp 1-80 (1998).
11. Masgrau, L., Basran, J., Hothi, P., Sutcliffe, M.J. & Scrutton, N.S. Hydrogen tunneling in quinoproteins. *Arch. Biochem. Biophys.* 428, 41-51 (2004).

12. Kay, C.W.M., Mennenga, B., Görisch, H. & Bittl, R. Substrate binding in quinoprotein ethanol dehydrogenase from *Pseudomonas aeruginosa* studied by electron-nuclear double resonance. *Proc. Natl. Acad. Sci.* 103(14), 5267-5272 (2006).
13. Anthony, C. & Williams, P. The structure and mechanism of methanol dehydrogenase. *Biochim. Biophys. Acta.* 1647, 18-23 (2003).
14. McIntire, W.S., Wemmer, D.E., Chistoserdov, A. & Lindstrom, M.E. A New Cofactor in a Prokaryotic Enzyme: Tryptophan Tryptophylquinone as the Redox Prosthetic Group in Methylamine Dehydrogenase. *Science.* 252, 817-824 (1991).
15. VanEngelen, M. R., Field, E. K., Gerlach, R., Lee, B. D., Apel, W. A., & Peyton, B. M. UO_2^{2+} speciation determines uranium toxicity and bioaccumulation in an environmental *Pseudomonas* sp. isolate. Accepted for publication in *Environ. Tox. Chem.*
16. Itoh, S., Kawakami, H. & Fukuzumi S. Modeling of the Chemistry of Quinoprotein Methanol Dehydrogenase. Oxidation of Methanol by Calcium Complex of Coenzyme PQQ via Addition-Elimination Mechanism. *J. Am. Chem. Soc.* 119, 439-440 (1997).
17. Dimitrijevic, N. M., Poluektov, Saponjic, Z. V. & Rajh T. Complex and Charge Transfer between TiO_2 and Pyrroloquinoline Quinone. *J. Phys. Chem. B.* 110, 25392-25398 (2006).
18. Noji, N. *et al.* Simple and Sensitive Method for Pyrroloquinoline quinone (PQQ) Analysis in Various Foods Using Liquid Chromatography/Electrospray-Ionization Tandem Mass Spectrometry. *J. Agric. Food Chem.* 55, 7258-7263 (2007).
19. Baston, G.M.N., Berry, J.A., Bond, K.A., Boulton, K.A., Brownsword, M. & Linklater, C.M. Effects of cellulosic degradation products on uranium sorption in the geosphere. *J. Alloy. Compound.* 213/214, 475-480 (1994).
20. Zhu, Z. & Davidson, V.L. Kinetic and chemical mechanisms for the effects of univalent cations on the spectral properties of aromatic amine dehydrogenase. *Biochem. J.* 329, 175-182 (1998).
21. Becke, A. D. Density-functional exchange-energy approximation with correct asymptotic behavior. *Phys. Rev. A: Gen. Phys.* 38, 3098-3100 (1988).
22. Perdew, J. P. Density-Functional Approximation for the Correlation-Energy of the Inhomogeneous Electron-Gas. *Phys. Rev. B: Cond. Mater.* 33, 8822-8824 (1986).

23. Hay, P. J. & Wadt, W. R. Ab initio effective core potentials for molecular calculations. Potentials for the transition metal atoms scandium to mercury. *J. Chem. Phys.* 82, 270-83 (1985).
24. Hay, P. J. & Wadt, W. R. Ab initio effective core potentials for molecular calculations. Potentials for potassium to gold including the outermost core orbitals. *J. Chem. Phys.* 82, 299-310 (1985).
25. Tomasi, J. & Persico, M. Molecular Interactions in Solution: An Overview of Methods Based on Continuous Distributions of the Solvent. *Chem. Rev.* 94, 2027-2094 (1994).
26. Cossi, M., Barone, V., Cammi, R. & Tomasi, J. Ab initio study of solvated molecules: a new implementation of the polarizable continuum model. *Chem. Phys. Lett.* 255, 327-335 (1996).
27. Frisch, M. J. et al. (ed. Gaussian, I.) (Gaussian, Inc., Wallingford, CT, 2004).
28. Di Toro, D. M. *et al.* Biotic Ligand Model of the Accute Toxicity of Metals. 1. Technical Basis. *Environ. Tox. Chem.* 20(10), 2383-2396 (2001).
29. Niyogi, S. & Wood C. M. Biotic Ligand Model, a Flexible Tool for Developing Site-Specific Water Quality Guidelines for Metals. *Environ. Sci. Technol.* 38(23), 6177-6192 (2004).
30. Webb, S. M., Fuller, C. C., Tebo B. M. & Bargar, J. R. Determination of Uranyl Incorporation into Biogenic Manganese Oxides Using X-ray Absorption Spectroscopy and Scattering. *Environ. Sci. Technol.* 40, 771-777 (2006).
31. Johnson, H.A. & Tebo, B.M. In vitro studies indicate a quinone is involved in bacterial Mn(II) oxidation. *Arch. Microbiol.* 189,59-69 (2008).
32. Manceau, A., Gorshkov, A. I., & Drits V. A. Structural chemistry on Mn, Fe, Co, and Ni in manganese hydrous oxides: Part II. Information from EXAFS spectroscopy and electron and X-ray diffraction. *Am. Mineral.* 77, 1144-1157 (1992).
33. Davis, J. A. & Curtis G. P. Application of Surface Complexation Modeling to Describe Uranium Adsorption and Retardation at the Uranium Mill Tailings Site at Naturita, Colorado. (NUREG/CR-6820) U.S. Nuclear Regulatory Commission. (2003).
34. Choy, C. C., Korfiatis, G. P. & Meng, X. Removal of depleted uranium from contaminated soils. *J. Hazard. Mat.* 136, 53-60 (2006).

35. Kasahara, T. & Sato, T. A new redox-cofactor vitamin for mammals. *Nature*. 422, 832 (2003).
36. Felton, L.M. & Anthony, C. Role of PQQ as a mammalian enzyme cofactor? *Nature*. 433, E10 (2005).
37. Bastide, P.J. & Bastide, P. 5-Hydroxytryptophan-decarboxylase and Monoamine Oxidase Activities in Kidneys of Rats and Mice after Experimental Uranyl Nitrate Intoxication. *Annales De Biologie Clinique*. 26(10-12), 1239-1245 (1968).

CONCLUSIONS AND FUTURE WORK

Concern over diminishing supplies and contributions to climate change have led the US and other nations to seek alternatives to fossil fuels for electricity generation. Among the alternatives receiving considerable attention, both in the US and around the world, is nuclear power. In the US, the Nuclear Power 2010 initiative was signed into law in 2002 and since then applications for 22 new nuclear power facilities have been received by the Nuclear Regulatory Commission (NRC) (Figure 14), which would add to the 104 reactors currently supplying 19.6% of the energy used in the US (3). According to the 2009 Annual Energy Outlook report prepared by the US Department of Energy (DOE), 13000 MW of nuclear generated capacity will be added by the year 2030 (1). This recent surge in interest in nuclear power has been declared an “atomic renaissance”, and represents the end of the 30 year period in which no new applications for nuclear reactors were submitted (4). This planned increase in nuclear power usage will inevitably lead to an increase in the amount of uranium (U) mined and processed. In 2008, nearly 2000 tons of U was removed from mines in the US, representing a 176% increase over the amount mined five years earlier (2).

This increase in U-associated activity will increase the likelihood of human and environmental exposure to U, and therefore understanding mechanisms of U toxicity will become more important. One mechanism was described in Chapter 4, and this knowledge should be applied in future investigations of U toxicity mechanisms. Specifically, the U binding site on PQQ is not a coordination environment unique to PQQ. As mentioned in Chapter 4, the catalytic centers of flavoproteins, derived from riboflavin, have similar



References

1. United States Department of Energy, Energy Information Administration. March, 2009. *Annual Energy Outlook 2009, With Projections to 2030*. Office of Integrated Analysis and Forecasting. Washington, DC 20585.
2. United States Department of Energy, Energy Information Administration. May, 2009. U. S. Uranium Mine Production and Number of Mines and Sources. From EIA-851A, *Domestic Uranium Production Report (2003-2008)*. Washington, DC 20585.
3. United States Nuclear Regulatory Commission. Location of Projected New Nuclear Power Reactors. <http://www.nrc.gov/reactors/new-reactors/col/new-reactor-map.html>. Accessed 5/21/09.
4. Atomic renaissance. (2007, September 8). *The Economist*, 384:71-73.

APPENDICES

APPENDIX A

RESPONSE OF *PSEUDOMONAS AERUGINOSA* PAO1 TO HIGH UO_2^{2+}
CONCENTRATIONS

Results were acquired with *Pseudomonas aeruginosa* PAO1 which will be discussed here, as they could lead to future work regarding metal toxicity response mechanisms. PAO1 was grown aerobically on ethanol in the presence of UO_2^{2+} , as described in Chapter 4. Consistent with the results shown in Chapter 4, 1 μM of UO_2^{2+} was able to significantly inhibit the growth of PAO1. Interestingly, increasing the UO_2^{2+} concentration to 10 or 50 μM lowered the toxicity of UO_2^{2+} to PAO1 (Figure 15). Additionally, growth of PAO1 in the presence of 10 or 50 μM UO_2^{2+} led to a change in media color from colorless to pale green. The absorbance spectrum of filtered media (0.2 μm) after growth in 50 μM UO_2^{2+} was obtained (Figure 16) and the peak at 395 nm indicates the presence of pyoverdins (2, 1). Pyoverdins are a class of siderophores produced by *Pseudomonas* spp., and are known to bind UO_2^{2+} (3). It can therefore be hypothesized that the production of pyoverdin by PAO1 represents a means of sequestering UO_2^{2+} in order to limit the bioavailability and toxicity of UO_2^{2+} . This defense mechanism would be consistent with the biotic ligand model (BLM) of acute metal toxicity which states that the toxicity of a given metal is mitigated in the presence competing ligands (4), which in this case is pyoverdin.

It can be further hypothesized that 1 μM UO_2^{2+} , while low enough to inhibit PAO1, is a concentration too low to induce the defense mechanism of pyoverdin production. This would explain why 1 μM UO_2^{2+} is more inhibitory than 10 or 50 μM UO_2^{2+} , concentrations which, while toxic, also induce the active detoxification mechanism of pyoverdin production. This hypothesis could be confirmed through a series of experiments aimed at comparing the transcriptomes of PAO1 cells grown in the

presence of 0, 1, and 50 μM UO_2^{2+} . The transcriptomes of cells grown in the presence of 0 and 50 μM UO_2^{2+} would serve as negative and positive controls, respectively, against which the transcriptome of the cells grown in the presence of 1 μM UO_2^{2+} would be compared. If the above hypothesis is correct, the transcriptomes of the cells grown with 0 and 1 μM UO_2^{2+} should be similar, and would not include transcripts of the *pvd* genes associated with pyoverdinin synthesis (6). These experiments could be performed using equipment and expertise available at Montana State University.

Another interesting feature of aerobic growth of PAO1 on ethanol in the presence of 50 μM UO_2^{2+} is the formation of visible, spherical cell clusters. These cells clusters are approximately 0.5 mm in diameter, and readily settle in unagitated media. Field emission scanning electron microscopy (FE-SEM) was used to investigate these clusters (Figures 17a-b). Images were captured at the Image and Chemical Analysis Laboratory (ICAL) at MSU using a JEOL 6100 FE-SEM with a LaB6 source. Digital images were captured using Rontec MultiImage software. Samples were air dried on silicon wafers prior to imaging.

Figures 17 a and b reveal PAO1 cells closely packed together and surrounded by smaller square shaped crystals approximately 100 - 200 nm is size. Figure 18 is an image of these precipitates at 19,000x magnification. Energy dispersive X-ray spectroscopy (EDS) was used to investigate the content of these precipitates (Figure 20a-c). The EDS system consisted of a NORAN SiLi detector driven by Rontec electronics and software.

The EDS spectrum of the Si wafer provided a negative control (Figure 20a) and only Si contributed significantly to the spectrum. Figure 20b is the EDS spectrum of a

region occupied by a monolayer of cells in the absence of precipitate, and served as a second negative control. Figure 20c is the EDS spectrum of a region containing cells and significant amounts of precipitate. The exact region is shown in Figure 19, which contains both the image of Figure 17a, and its corresponding electron density image, where bright regions correspond to regions of high electron density.

When focused on a region lacking significant amounts of precipitate, the EDS spectrum of PAO1 cells (Figure 20b) revealed the presence of 5.55% U (on an atomic percent basis). The presence of U was expected given the ability of PAO1 to readily accumulate UO_2^{2+} (5). The EDS spectrum of a region containing both cells and precipitate, as highlighted in Figure 20c, revealed the presence of significantly more U compared to the precipitate free spectrum. U accounted for 8.8% of the elemental composition (on an atomic percent basis), representing a 60% increase over the precipitate free spectrum. Also, unlike the two control spectra, a significant amount of Ca^{2+} was detected. It is possible that the precipitates consist of UO_2^{2+} - pyoverdine complexes, perhaps similar to the complexes described in Moll, et al. (2008) (3). This would help explain why the production of pyoverdine coincides with lower sensitivity of PAO1 to UO_2^{2+} , as demonstrated in Figures 14 and 15, and would be consistent with the hypothesis that the production of pyoverdine by PAO1 represents a means of sequestering UO_2^{2+} in order to limit the bioavailability and toxicity of UO_2^{2+} .

An extension of this work could focus on isolating the pyoverdine molecule, elucidating its structure, and identifying the UO_2^{2+} binding sites using published protocols (3). Identifying potential metal binding sites could also explain the presence of

Ca^{2+} in the precipitates, since in addition to UO_2^{2+} it appears likely that the pyoverdine also efficiently binds Ca^{2+} . In combination with the transcriptomic work proposed earlier, this information could provide very interesting insights into metal toxicity response mechanisms of PAO1.

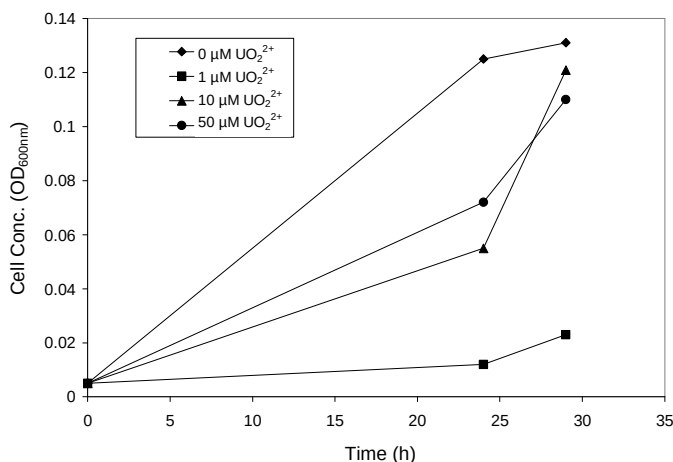


Figure 15. UO_2^{2+} induced growth inhibition of PAO1 during aerobic growth on ethanol. Between the three UO_2^{2+} concentrations used (1, 10, and 50 μM), 1 μM UO_2^{2+} was the most inhibitory

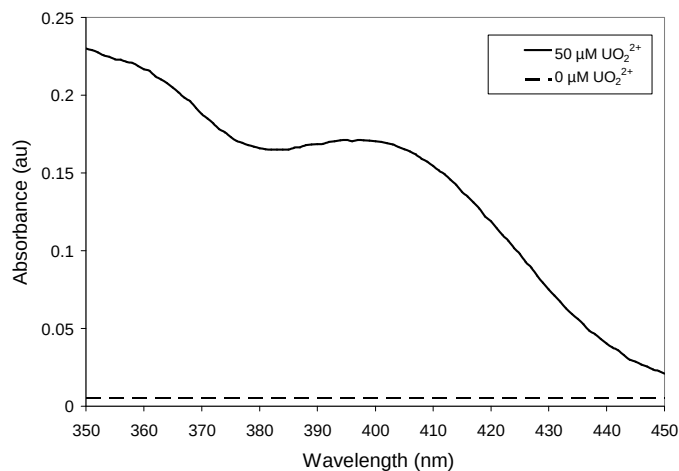


Figure 16. Absorbance spectra of filtered media (0.2 μm) after aerobic growth of PAO1 on ethanol in the presence and absence of 50 μM UO_2^{2+} . The absorbance max near 400 nm produced in the presence of 50 μM UO_2^{2+} is typical of pyoverdins produced by *Pseudomonas* spp.

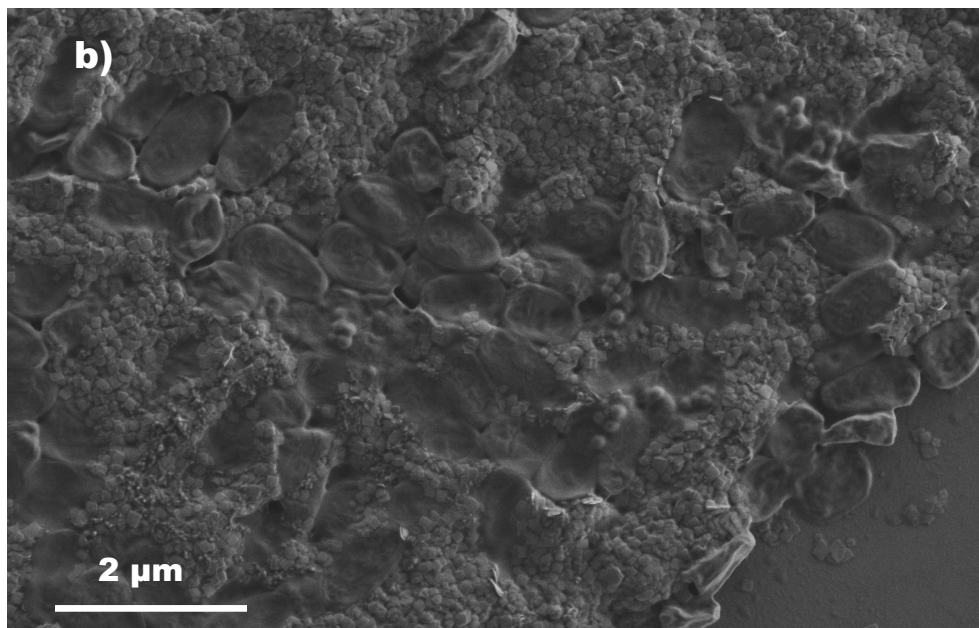
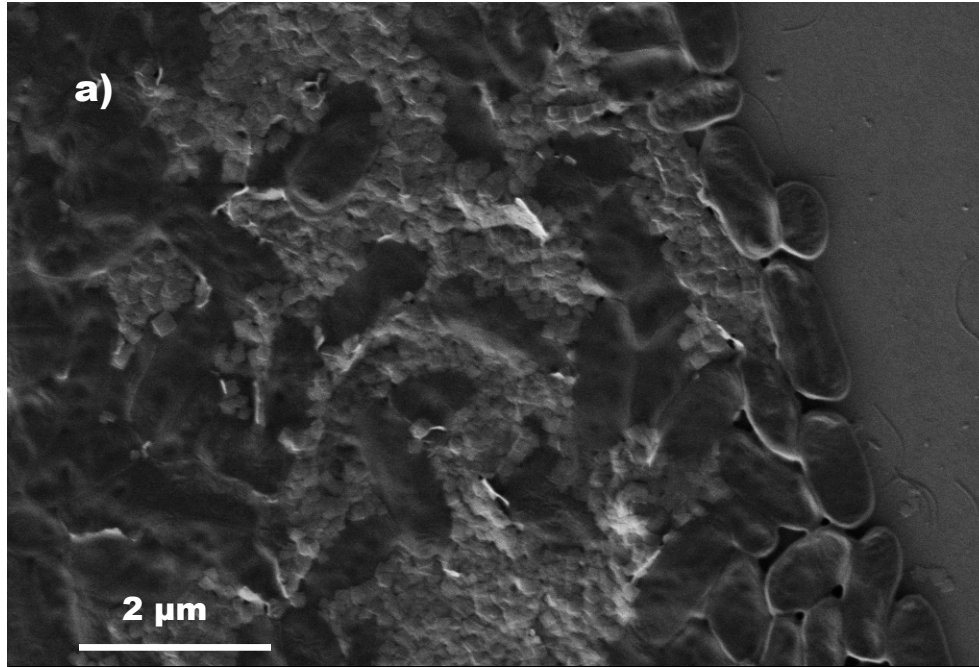


Figure 17(a-b). FE-SEM images of PAO1 cell clusters formed during aerobic growth on ethanol in the presence of 50 μM UO_2^{2+} . Images are magnified 9,000x.

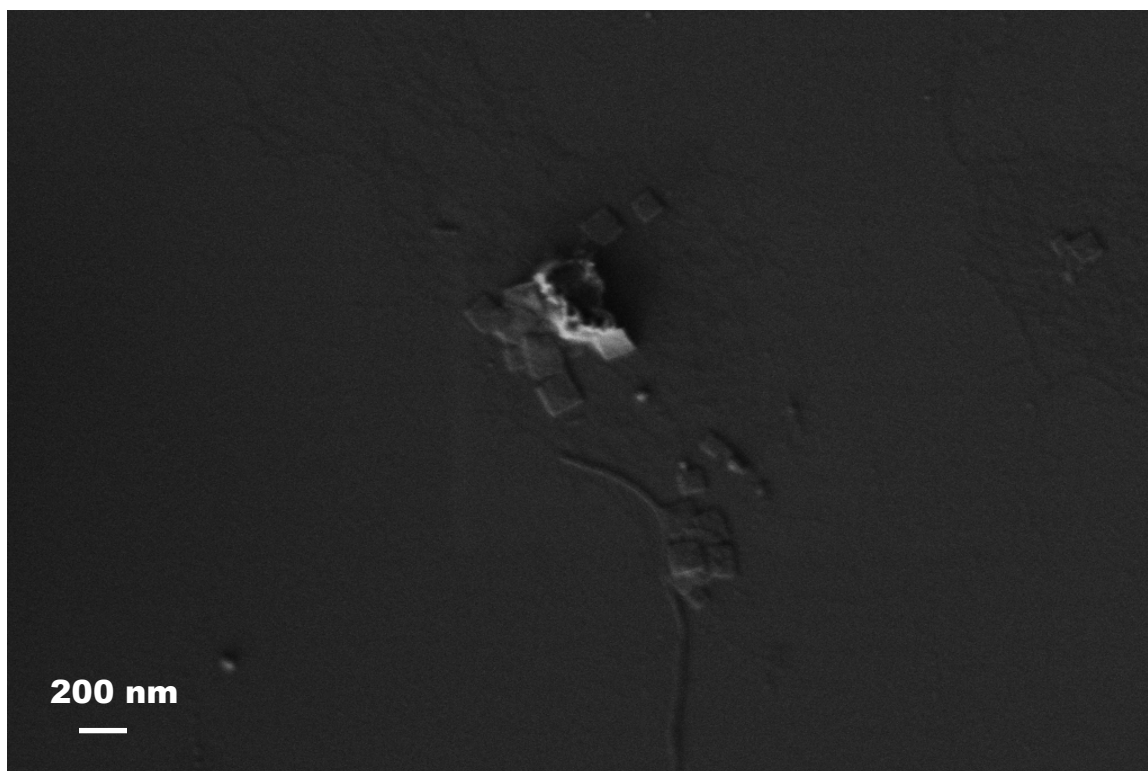


Figure 18. FE-SEM image of square shaped crystals found within the PAO1 cell clusters. Crystals are flat and range in size from 100 – 200 nm. Image is magnified 19,000x.

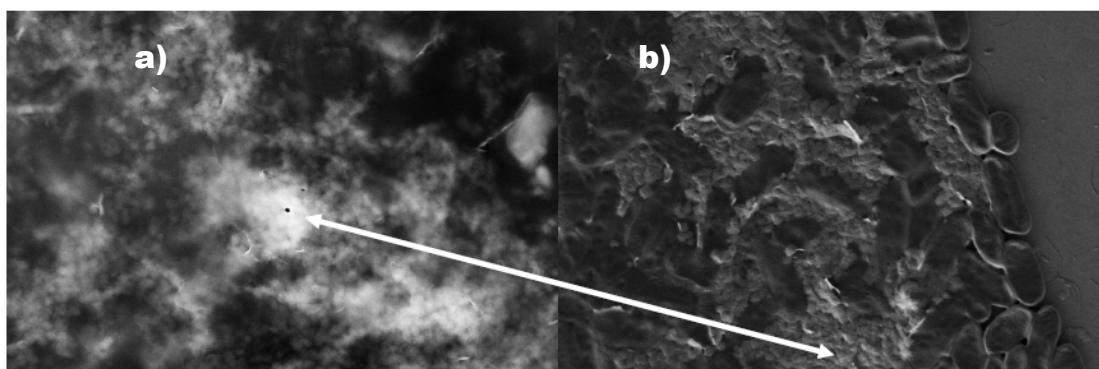
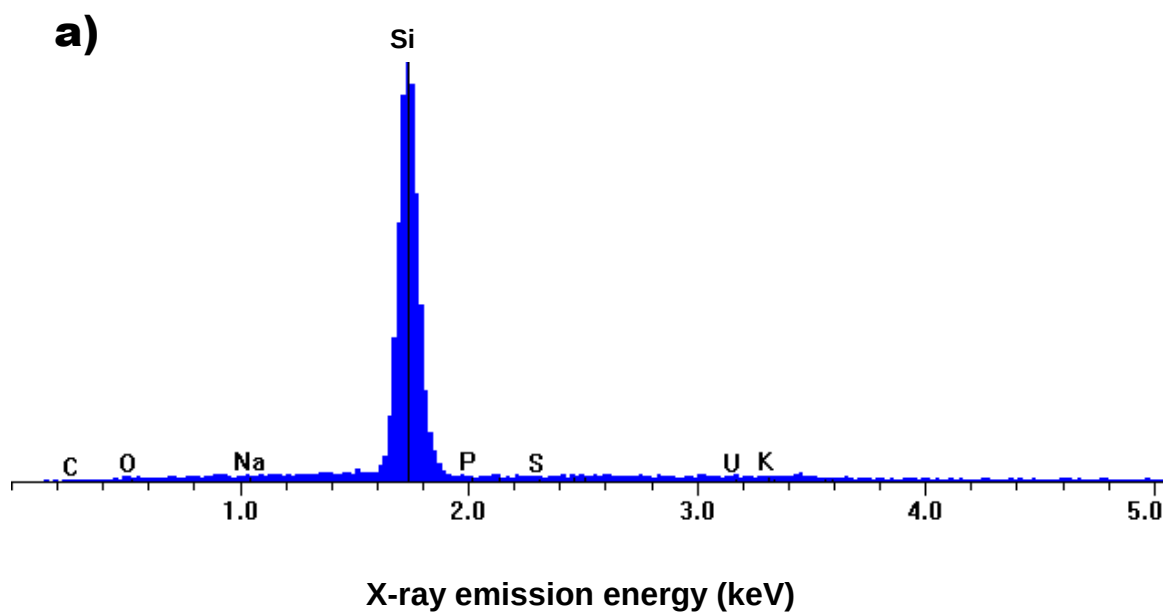
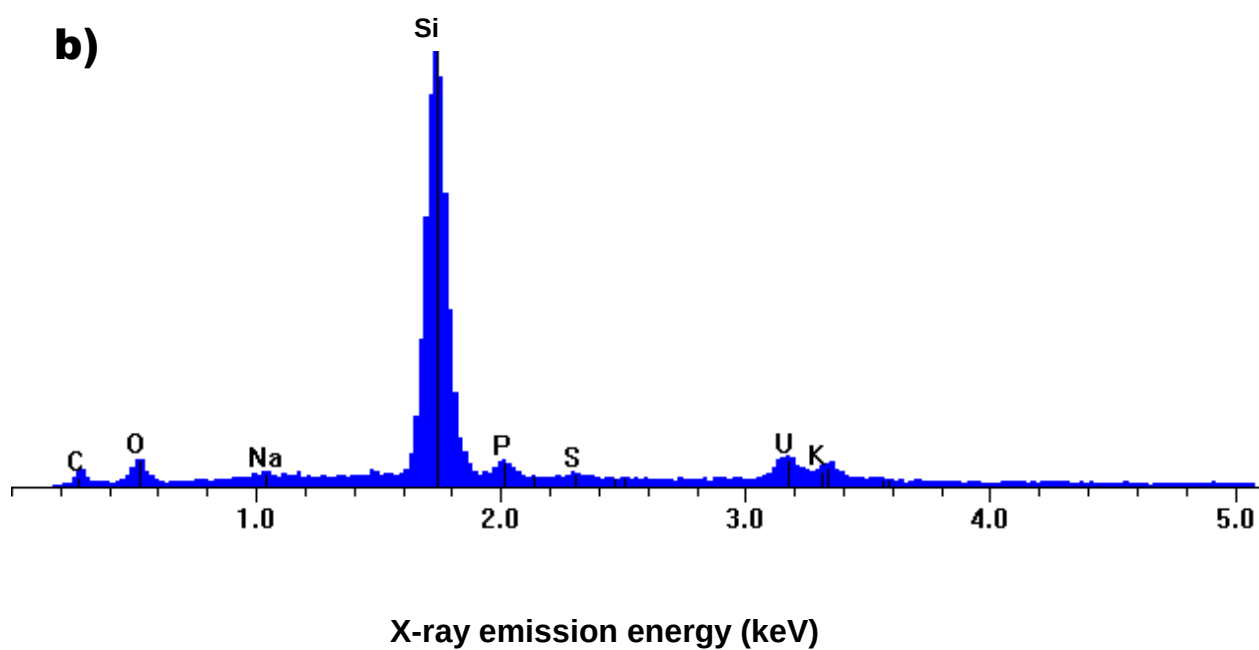


Figure 19a. Electron density image showing source of EDS data in Figure 6c. Image is magnified 10,650x. Figure 7b is the same image in Figure 4a, and the white line points to the exact region of data collection, and connects identical regions on the two images.



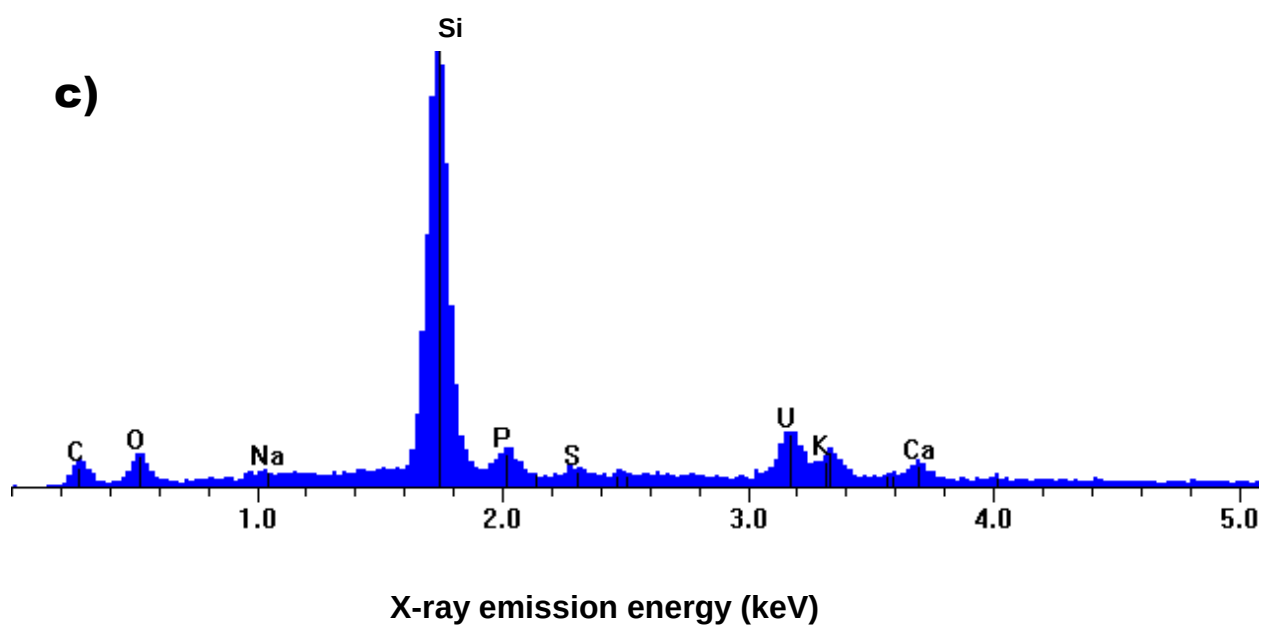
Element	Wt%	At%
C	0.06	0.14
O	1.26	2.19
Na	0.03	0.03
Si	98.65	97.63
P	0	0
K	0	0
U	0	0
S	0	0
Total	100	100

Figure 20a. EDS spectrum of Si wafer and corresponding elemental distribution data (on both a weight percent and atomic percent basis).



Element	Wt%	At%
C	3.14	9.19
O	12.39	27.2
Na	0.82	1.26
Si	41.84	52.33
P	2.26	2.57
K	0.95	0.86
U	37.63	5.55
S	0.95	1.05
Total	100	100

Figure 20b. EDS spectrum of PAO1 cells and corresponding elemental distribution data (on both a weight percent and atomic percent basis).



Element	Wt%	At%
C	2.85	9.96
O	11.13	29.2
Na	0.5	0.92
Si	28.66	42.85
P	2.01	2.72
K	1.2	1.29
U	49.85	8.8
S	1.04	1.36
Ca	2.76	2.89
Total	100	100

Figure 20c. EDS spectrum of region containing both PAO1 cells and precipitates. Elemental distribution data (on both a weight percent and atomic percent basis) is included in the table.

References

1. Bultreys A, Gheysen I, Wathelet B, Maraite H, de Hoffmann E. 2003. High-Performance Liquid Chromatography Analyses of Pyoverdine Siderophores Differentiate among Phytopathogenic Fluorescent *Pseudomonas* Species. *Appl. Environ. Microbiol.* 69(2):1143-1153.
2. Cox CD, Adams P. 1985. Siderophore Activity of Pyoverdine for *Pseudomonas aeruginosa*. *Infect. Immun.* 48(1):130-138.
3. Moll H, Glorius M, Bernhard G, Johnsson A, Pedersen K, Schäfer M, Budzikiewicz H. 2008. Characterization of Pyoverdins Secreted by a Subsurface Strain of *Pseudomonas fluorescens* and Their Interactions with Uranium(VI). *Geomicrobiol. J.* 25:157-166.
4. Niyogi S, Wood CM. 2004. Biotic Ligand Model, a Flexible Tool for Developing Site-Specific Water Quality Guidelines for Metals. *Environ. Sci. Technol.* 38(23):6177-6192.
5. Strandberg GW, Shumate II SE, Parrott Jr JR. 1981. Microbial Cells as Biosorbents for Heavy Metals: Accumulation of Uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 41(1):237-245.
6. Tsuda M, Miyazaki H, Nakazawa T. 1995. Genetic and Physical Mapping of Genes Involved in Pyoverdine Production in *Pseudomonas aeruginosa* PAO. *J. Bacteriol.* 177(2):423-431.

APPENDIX B

ISOLATE A GROWTH DATA IN LOW BICARBONATE MEDIA

This appendix contains tabulated OD_{600nm} measurements used to generate Figure 1(a-d) of Chapter 2. Media composition and growth conditions can found in Chapter 2. Tables 5, 6, 7, and 8 contain the data from the butyrate, dextrose, ethanol, and lactate systems, respectively. OD_{600nm} to mg-cell protein/L conversions were made using the standard curves shown in Appendix C. Experiments were performed in triplicate, and data points correspond to the average of triplicate measurements. Reported errors correspond to 95% confidence intervals.

Table 5. OD_{600nm} measurements and conversion to mg-cell protein/L conversions during growth of Isolate A on butyrate in the presence of UO₂²⁺.

Butyrate 0 μM UO ₂ ²⁺								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg-protein/L	95% CI
0	0.099	0.098	0.1	0.099	0.001	0.01	3.11	0.02
4	0.107	0.106	0.111	0.107	0.001	0.01	3.60	0.02
8	0.136	0.138	0.136	0.137	0.001	0.01	5.49	0.06
12	0.203	0.201	0.199	0.202	0.001	0.01	9.52	0.07
16	0.319	0.316	0.314	0.318	0.002	0.01	16.7	0.11
20	0.329	0.33	0.328	0.330	0.001	0.00	17.4	0.04
24	0.312	0.319	0.315	0.316	0.005	0.02	16.6	0.26
28	0.304	0.305	0.301	0.305	0.001	0.00	15.9	0.04

50 μM UO ₂ ²⁺								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg-protein/L	95% CI
0	0.103	0.101	0.102	0.102	0.001	0.01	3.32	0.05
4	0.107	0.108	0.108	0.108	0.001	0.01	3.67	0.02
8	0.119	0.136	0.127	0.128	0.012	0.09	4.91	0.46
12	0.16	0.195	0.177	0.178	0.025	0.14	8.01	1.12
16	0.243	0.278	0.255	0.261	0.025	0.1	13.2	1.25
20	0.335	0.309	0.326	0.322	0.018	0.1	17.0	0.97
24	0.338	0.297	0.315	0.318	0.029	0.1	16.7	1.52
28	0.314	0.288	0.303	0.301	0.018	0.1	15.7	0.96

100 μM UO ₂ ²⁺								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg-protein/L	95% CI
0	0.102	0.105	0.103	0.1035	0.00	0.02	3.42	0.07
4	0.107	0.107	0.106	0.107	0.00	0.00	3.63	0.00
8	0.134	0.133	0.129	0.1335	0.00	0.01	5.28	0.03
12	0.179	0.167	0.175	0.173	0.01	0.05	7.73	0.38
16	0.218	0.22	0.219	0.219	0.00	0.01	10.6	0.07
20	0.275	0.283	0.282	0.279	0.01	0.02	14.3	0.29
24	0.309	0.329	0.318	0.319	0.01	0.04	16.8	0.74
28	0.3	0.323	0.305	0.3115	0.02	0.05	16.3	0.85

125 μM UO_2^{2+}								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg- protein/L	95% CI
0	0.104	0.106	0.105	0.105	0.00	0.01	3.51	0.05
4	0.108	0.11	0.109	0.109	0.00	0.01	3.76	0.05
8	0.134	0.136	0.135	0.135	0.00	0.01	5.37	0.06
12	0.159	0.144	0.154	0.1515	0.01	0.07	6.39	0.45
16	0.175	0.156	0.161	0.1655	0.01	0.08	7.26	0.59
20	0.202	0.166	0.187	0.184	0.03	0.14	8.41	1.16
24	0.209	0.167	0.189	0.188	0.03	0.16	8.66	1.37
28	0.199	0.158	0.176	0.1785	0.03	0.16	8.07	1.31

150 μM UO_2^{2+}								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg- protein/L	95% CI
0	0.106	0.108	0.101	0.107	0.0014	0.013	3.63	0.05
4	0.107	0.113	0.104	0.11	0.0042	0.039	3.82	0.15
8	0.109	0.109	0.103	0.109	0.0037	0.034	3.76	0.13
12	0.113	0.111	0.105	0.112	0.0014	0.013	3.94	0.05
16	0.117	0.115	0.109	0.116	0.0014	0.012	4.19	0.05
20	0.116	0.123	0.113	0.1195	0.0049	0.041	4.41	0.18
24	0.122	0.119	0.113	0.1205	0.0021	0.018	4.47	0.08
28	0.125	0.119	0.115	0.122	0.0042	0.035	4.56	0.16

Table 6. OD_{600nm} measurements and conversion to mg-cell protein/L conversions during growth of Isolate A on dextrose in the presence of UO₂²⁺.

Dextrose 0 μM UO₂²⁺								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg-protein/L	95% CI
0	0.104	0.104	0.104	0.104	0	0.00	3.45	0.00
4	0.128	0.13	0.127	0.128	0.002	0.01	4.96	0.06
8	0.25	0.24	0.253	0.248	0.007	0.03	12.4	0.34
11	0.355	0.326	0.371	0.351	0.023	0.07	18.7	1.22
16	0.447	0.442	0.448	0.446	0.003	0.01	24.6	0.18
20	0.438	0.434	0.424	0.432	0.007	0.02	23.8	0.40
24	0.341	0.329	0.333	0.334	0.006	0.02	17.7	0.32

50 μM UO₂²⁺								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg-protein/L	95% CI
0	0.108	0.116	0.109	0.111	0.004	0.04	3.88	0.15
4	0.119	0.113	0.12	0.117	0.004	0.03	4.27	0.14
8	0.183	0.15	0.155	0.163	0.018	0.11	7.09	0.77
11	0.266	0.245	0.245	0.252	0.012	0.05	12.6	0.61
16	0.453	0.433	0.435	0.440	0.011	0.03	24.3	0.61
20	0.413	0.421	0.414	0.416	0.004	0.01	22.8	0.24
24	0.323	0.32	0.319	0.321	0.002	0.01	16.9	0.11

100 μM UO₂²⁺								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg-protein/L	95% CI
0	0.115	0.124	0.124	0.121	0.005	0.04	4.50	0.19
4	0.125	0.13	0.12	0.125	0.005	0.04	4.75	0.19
8	0.195	0.192	0.162	0.183	0.018	0.10	8.35	0.83
11	0.235	0.233	0.202	0.223	0.019	0.08	10.8	0.90
16	0.401	0.413	0.378	0.397	0.018	0.04	21.6	0.97
20	0.476	0.467	0.458	0.467	0.009	0.02	26.0	0.50
24	0.37	0.355	0.364	0.363	0.008	0.02	19.5	0.41

125 μM UO_2^{2+}

Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg- protein/L	95% CI
0	0.126	0.125	0.127	0.126	0.001	0.01	4.81	0.04
4	0.136	0.137	0.139	0.137	0.002	0.01	5.51	0.06
8	0.169	0.168	0.158	0.165	0.006	0.04	7.23	0.27
11	0.186	0.182	0.166	0.178	0.011	0.06	8.04	0.48
16	0.256	0.248	0.217	0.240	0.021	0.09	11.9	1.02
20	0.269	0.271	0.232	0.257	0.022	0.09	13.0	1.11
24	0.253	0.234	0.202	0.230	0.026	0.11	11.2	1.26

150 μM UO_2^{2+}

Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg- protein/L	95% CI
0	0.112	0.118	0.112	0.114	0.003	0.03	4.07	0.12
4	0.117	0.119	0.117	0.118	0.001	0.01	4.30	0.04
8	0.117	0.114	0.116	0.116	0.002	0.01	4.17	0.06
11	0.117	0.115	0.116	0.116	0.001	0.01	4.19	0.04
16	0.131	0.128	0.13	0.130	0.002	0.01	5.04	0.06
20	0.131	0.126	0.13	0.129	0.003	0.02	5.00	0.10
24	0.117	0.113	0.115	0.115	0.002	0.02	4.13	0.07

Table 7. OD_{600nm} measurements and conversion to mg-cell protein/L conversions during growth of Isolate A on ethanol in the presence of UO₂²⁺.

Ethanol 0 μM UO₂²⁺								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg-protein/L	95% CI
0	0.101	0.101	0.103	0.102	0.002	0.023	3.3	0
4	0.105	0.107	0.107	0.106	0.003	0.027	3.6	0.10
8	0.144	0.156	0.155	0.152	0.017	0.112	6.3	0.71
10	0.188	0.202	0.195	0.195	0.020	0.102	9.1	0.92
11	0.25	0.263	0.255	0.256	0.018	0.072	12.9	0.92
14	0.339	0.339	0.337	0.338	0.002	0.007	18.0	0.12
16	0.383	0.383	0.388	0.385	0.006	0.015	20.7	0.31
18	0.364	0.383	0.37	0.372	0.027	0.072	20.2	1.45
20	0.349	0.344	0.339	0.344	0.007	0.021	18.5	0.38
27	0.328	0.334	0.333	0.332	0.008	0.026	17.5	0.45

0.1 μM UO₂²⁺								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg-protein/L	95% CI
0	0.1	0.101	0.103	0.101	0.001	0.007	3.2	0.0
4	0.106	0.108	0.109	0.108	0.001	0.013	3.6	0.0
8	0.137	0.131	0.132	0.133	0.004	0.032	5.3	0.2
10	0.169	0.159	0.155	0.161	0.007	0.044	7.2	0.3
11	0.21	0.19	0.201	0.200	0.014	0.071	9.4	0.7
14	0.295	0.269	0.284	0.283	0.018	0.065	14.5	0.9
16	0.339	0.334	0.333	0.335	0.004	0.011	17.9	0.2
18	0.381	0.373	0.372	0.375	0.006	0.015	20.4	0.3
20	0.357	0.384	0.369	0.370	0.019	0.052	20.0	1.0
27	0.327	0.333	0.333	0.331	0.004	0.013	17.5	0.2

0.15 μM UO₂²⁺								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg-protein/L	95% CI
0	0.102	0.102	0.101	0.102	0.001	0.011	3.32	0.04
4	0.106	0.107	0.105	0.106	0.001	0.013	3.60	0.05
8	0.116	0.116	0.112	0.115	0.005	0.040	4.19	0.17
10	0.124	0.15	0.131	0.135	0.037	0.272	5.49	1.47
11	0.133	0.139	0.133	0.135	0.008	0.063	5.43	0.34
14	0.146	0.157	0.152	0.152	0.016	0.103	6.39	0.66
16	0.162	0.172	0.165	0.166	0.014	0.085	7.35	0.62
18	0.185	0.215	0.205	0.202	0.042	0.210	9.40	1.99
20	0.195	0.204	0.207	0.202	0.013	0.063	9.37	0.60
27	0.316	0.301	0.31	0.309	0.021	0.069	16.1	1.11
30	0.338	0.328	0.333	0.333	0.014	0.042	17.6	0.75
45	0.327	0.311	0.318	0.319	0.023	0.071	16.8	1.19

0.25 μM UO_2^{2+}								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg- protein/L	95% CI
0	0.109	0.103	0.11	0.107	0.008	0.079	3.57	0.29
4	0.108	0.107	0.108	0.108	0.001	0.013	3.67	0.05
8	0.115	0.115	0.112	0.114	0.003	0.030	4.13	0.13
10	0.123	0.121	0.125	0.123	0.003	0.023	4.56	0.11
11	0.128	0.125	0.122	0.125	0.004	0.034	4.84	0.16
14	0.139	0.135	0.138	0.137	0.006	0.041	5.49	0.23
16	0.15	0.143	0.141	0.145	0.010	0.068	6.08	0.41
18	0.162	0.152	0.151	0.155	0.014	0.091	6.73	0.61
20	0.177	0.164	0.184	0.175	0.018	0.105	7.57	0.82
27	0.283	0.268	0.288	0.280	0.021	0.076	14.1	1.08
30	0.319	0.316	0.32	0.318	0.004	0.013	16.7	0.22
45	0.36	0.367	0.355	0.361	0.010	0.027	19.5	0.53

0.5 μM UO_2^{2+}								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg- protein/L	95% CI
0	0.101	0.101	0.1	0.101	0.001	0.006	3.24	0.02
4	0.104	0.104	0.103	0.104	0.001	0.006	3.43	0.02
6	0.104	0.104	0.104	0.104	0.000	0.000	3.45	0.00
9	0.104	0.107	0.105	0.105	0.002	0.015	3.53	0.05
12	0.107	0.106	0.11	0.108	0.002	0.019	3.68	0.07
14	0.108	0.107	0.114	0.110	0.004	0.035	3.80	0.13
16	0.111	0.109	0.118	0.113	0.005	0.042	3.99	0.17
18	0.112	0.109	0.12	0.114	0.006	0.050	4.05	0.20
20	0.119	0.113	0.125	0.119	0.006	0.050	4.38	0.22
27	0.129	0.122	0.142	0.131	0.010	0.077	5.12	0.40
30.5	0.166	0.13	0.158	0.151	0.019	0.125	6.38	0.80
33.5	0.142	0.138	0.168	0.149	0.016	0.109	6.26	0.68
51	0.238	0.192	0.293	0.241	0.051	0.210	11.9	2.51

1 μM UO_2^{2+}								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg- protein/L	95% CI
0	0.104	0.101	0.1	0.102	0.002	0.020	3.30	0.07
4	0.104	0.103	0.104	0.104	0.001	0.006	3.43	0.02
6	0.103	0.107	0.106	0.105	0.002	0.020	3.53	0.07
9	0.103	0.103	0.103	0.103	0.000	0.000	3.39	0.00
12	0.105	0.107	0.103	0.105	0.002	0.019	3.51	0.07
14	0.101	0.11	0.104	0.105	0.005	0.044	3.51	0.15
16	0.106	0.115	0.105	0.109	0.006	0.051	3.74	0.19
18	0.105	0.118	0.105	0.109	0.008	0.069	3.78	0.26
20	0.105	0.121	0.107	0.111	0.009	0.079	3.88	0.30
27	0.109	0.136	0.112	0.119	0.015	0.124	4.38	0.54
30.5	0.147	0.146	0.115	0.136	0.018	0.134	5.43	0.73
33.5	0.111	0.155	0.118	0.128	0.024	0.185	4.94	0.91
51	0.144	0.128	0.151	0.143	0.012	0.082	5.89	1.37

Table 8. $\text{OD}_{600\text{nm}}$ measurements and conversion to mg-cell protein/L conversions during growth of Isolate A on lactate in the presence of UO_2^{2+} .

Lactate 0 μM UO_2^{2+}								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg- protein/L	95% CI
0	0.119	0.118	0.118	0.118	0.001	0.01	4.34	0.04
4	0.152	0.161	0.15	0.154	0.012	0.08	6.57	0.50
6	0.189	0.193	0.194	0.192	0.005	0.03	8.90	0.25
8	0.29	0.27	0.267	0.276	0.025	0.09	14.1	1.28
10	0.334	0.315	0.313	0.321	0.023	0.07	16.9	1.22
14	0.309	0.333	0.32	0.321	0.024	0.07	16.9	1.26
18	0.339	0.33	0.33	0.333	0.010	0.03	17.6	0.55
22	0.326	0.325	0.328	0.326	0.003	0.01	17.2	0.16

50 μM UO_2^{2+}								
Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg- protein/L	95% CI
0	0.12	0.132	0.117	0.123	0.016	0.13	4.63	0.60
4	0.156	0.155	0.155	0.155	0.001	0.01	6.63	0.05
6	0.189	0.194	0.193	0.192	0.005	0.03	8.90	0.25
8	0.25	0.238	0.239	0.242	0.013	0.05	12.0	0.66
10	0.3	0.285	0.279	0.288	0.022	0.08	14.9	1.12
14	0.346	0.349	0.355	0.350	0.009	0.03	18.7	0.49
18	0.316	0.31	0.304	0.310	0.012	0.04	16.2	0.63
22	0.305	0.299	0.298	0.301	0.008	0.03	15.6	0.39

100 μM UO_2^{2+}

Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg-protein/L	95% CI
0	0.124	0.132	0.118	0.125	0.014	0.11	4.73	0.53
4	0.153	0.157	0.156	0.155	0.004	0.03	6.63	0.18
6	0.166	0.165	0.166	0.166	0.001	0.01	7.27	0.05
8	0.182	0.17	0.171	0.174	0.013	0.08	7.81	0.60
10	0.189	0.18	0.176	0.182	0.013	0.07	8.26	0.61
14	0.198	0.196	0.167	0.187	0.035	0.19	8.59	1.59
18	0.204	0.193	0.196	0.198	0.011	0.06	9.26	0.53
22	0.208	0.2	0.199	0.202	0.010	0.05	9.54	0.47

125 μM UO_2^{2+}

Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg-protein/L	95% CI
0	0.123	0.129	0.117	0.123	0.012	0.10	4.63	0.45
4	0.143	0.143	0.143	0.143	0.000	0.00	5.87	0.00
6	0.144	0.143	0.145	0.144	0.002	0.01	5.93	0.08
8	0.145	0.144	0.145	0.145	0.001	0.01	5.97	0.05
10	0.149	0.148	0.146	0.148	0.003	0.02	6.16	0.13
14	0.16	0.158	0.159	0.159	0.002	0.01	6.86	0.09
18	0.156	0.157	0.157	0.157	0.001	0.01	6.71	0.05
22	0.157	0.156	0.156	0.156	0.001	0.01	6.69	0.05

150 μM UO_2^{2+}

Time (h)	1	2	3	ave	95% CI	rel 95% CI	mg-protein/L	95% CI
0	0.121	0.126	0.12	0.122	0.006	0.05	4.58	0.24
4	0.128	0.126	0.127	0.127	0.002	0.02	4.87	0.08
6	0.124	0.124	0.129	0.126	0.006	0.05	4.79	0.22
8	0.124	0.124	0.126	0.125	0.002	0.02	4.73	0.09
10	0.124	0.127	0.125	0.125	0.003	0.02	4.77	0.12
14	0.123	0.124	0.124	0.124	0.001	0.01	4.67	0.04
18	0.121	0.121	0.124	0.122	0.003	0.03	4.56	0.13
22	0.124	0.123	0.124	0.124	0.001	0.01	4.67	0.04

APPENDIX C

GROWTH CURVES OF ISOLATE A IN THE PRESENCE OF UO_2^{2+} IN HIGH
BICARBONATE MEDIA

Growth curves of Isolate A in the presence of UO_2^{2+} and excess bicarbonate, used to generate Figure 5 in Chapter 2, are presented in this appendix, along with all corresponding $\text{OD}_{600\text{nm}}$ measurements. Cells were grown either on butyrate, dextrose, ethanol, or lactate in sealed serum bottles in which 10 mM NaHCO_3 was added, as described in Chapter 3. UO_2^{2+} concentrations ranged from 0 - 200 μM . Figures 21-24 and Tables 9-12 contain data derived from the butyrate, dextrose, ethanol, and lactate systems, respectively. Data points represent the average of triplicate measurements, and error bars correspond to 95% confidence intervals.

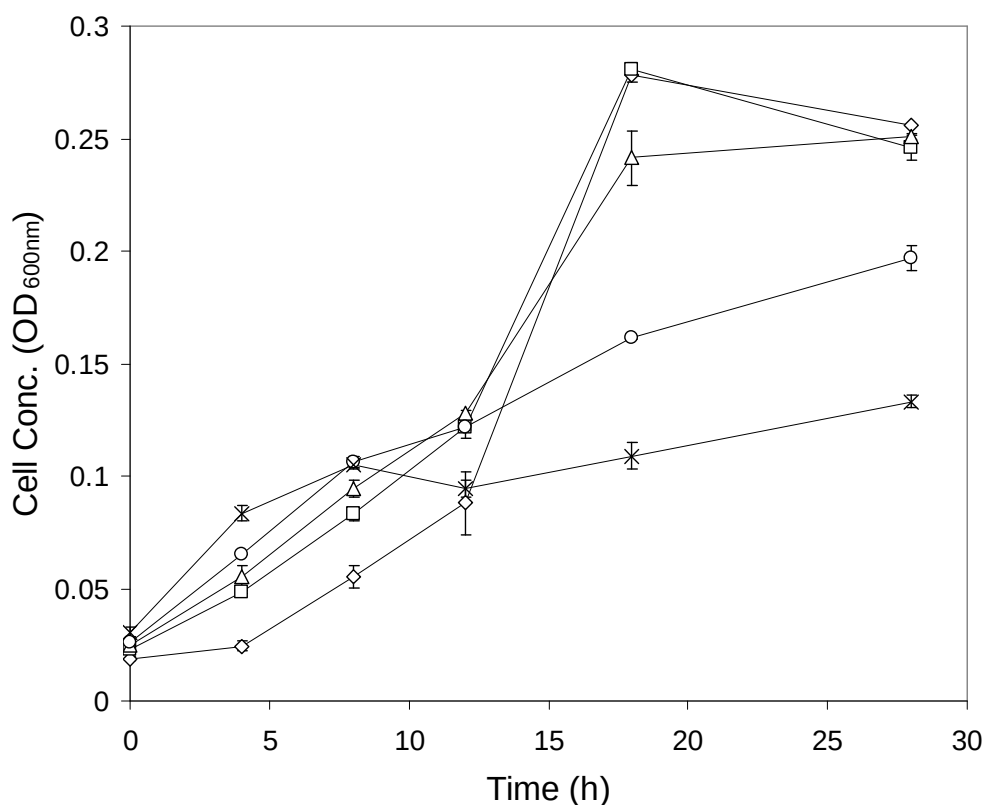


Figure 21. Growth curves of Isolate A growing on butyrate in the presence of UO_2^{2+} and excess carbonate ($\diamond$ = UO_2^{2+} free, $\square$ = 50 μM UO_2^{2+} , Δ = 100 μM UO_2^{2+} , $\circ$ = 150 μM UO_2^{2+} , and $\times$ = 200 μM UO_2^{2+}). Data points represent the average of triplicate measurements, and error bars correspond to 95% confidence intervals.

Table 9. OD_{600nm} values obtained during the growth of Isolate A on butyrate in the presence of excess carbonate and UO₂²⁺. Average values correspond to the average of triplicate measurements, and errors are reported as 95% confidence intervals.

Butyrate	0 μM UO_2^{2+}					50 μM UO_2^{2+}				
Time (h)	1	2	3	ave	95% CI	1	2	3	ave	95% CI
0	0.019	0.018	0.02	0.019	0.002	0.025	0.021	0.2	0.082	0.204
4	0.023	0.026	0.025	0.025	0.003	0.048	0.049	0.044	0.047	0.005
8	0.052	0.059	0.055	0.055	0.007	0.081	0.085	0.082	0.083	0.004
12	0.078	0.098	0.075	0.084	0.025	0.118	0.125	0.12	0.121	0.007
18	0.276	0.28	0.269	0.28	0.011	0.279	0.283	0.275	0.279	0.008
28	0.256	0.256	0.266	0.26	0.012	0.25	0.242	0.25	0.247	0.009

100 μM UO_2^{2+}						150 μM UO_2^{2+}				
Time (h)	1	2	3	ave	95% CI	1	2	3	ave	95% CI
0	0.025	0.025	0.027	0.026	0.002	0.025	0.027	0.023	0.025	0.004
4	0.059	0.052	0.052	0.054	0.008	0.065	0.065	0.067	0.066	0.002
8	0.097	0.092	0.091	0.093	0.006	0.108	0.104	0.101	0.104	0.007
12	0.129	0.127	0.128	0.128	0.002	0.121	0.122	0.119	0.121	0.003
18	0.233	0.25	0.244	0.242	0.017	0.162	0.161	0.158	0.160	0.004
28	0.25	0.252	0.252	0.251	0.002	0.201	0.193	0.19	0.195	0.011

200 μM UO_2^{2+}					
Time (h)	1	2	3	ave	95% CI
0	0.032	0.029	0.021	0.027	0.011
4	0.086	0.081	0.08	0.082	0.006
8	0.104	0.106	0.101	0.104	0.005
12	0.092	0.097	0.09	0.093	0.007
18	0.105	0.113	0.112	0.11	0.009
28	0.131	0.135	0.137	0.13	0.006

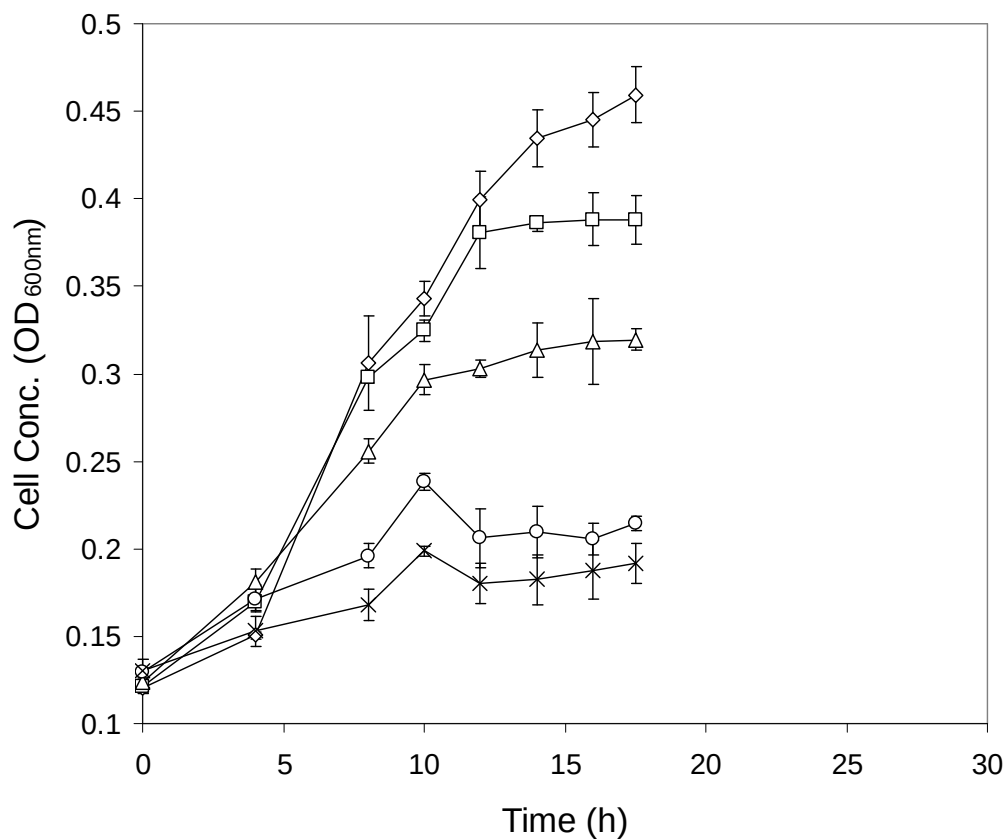


Figure 22. Growth curves of Isolate A growing on dextrose in the presence of UO_2^{2+} and excess carbonate ($\diamond = \text{UO}_2^{2+}$ free, $\square = 50 \mu\text{M UO}_2^{2+}$, $\Delta = 100 \mu\text{M UO}_2^{2+}$, $\circ = 150 \mu\text{M UO}_2^{2+}$, and $\times = 200 \mu\text{M UO}_2^{2+}$). Data points represent the average of triplicate measurements, and error bars correspond to 95% confidence intervals.

Table 10. OD_{600nm} values obtained during the growth of Isolate A on dextrose in the presence of excess carbonate and UO₂²⁺. Average values correspond to the average of triplicate measurements, and errors are reported as 95% confidence intervals.

Dextrose 0 μM UO ₂ ²⁺						50 μM UO ₂ ²⁺				
Time (h)	1	2	3	ave	95% CI	1	2	3	ave	95% CI
0	0.118	0.123	0.119	0.12	0.005	0.127	0.129	0.127	0.128	0.002
4	0.152	0.149	0.148	0.150	0.004	0.175	0.181	0.177	0.178	0.006
8	0.287	0.325	0.296	0.303	0.040	0.316	0.311	0.31	0.312	0.006
10	0.35	0.336	0.344	0.343	0.014	0.337	0.346	0.339	0.341	0.009
12	0.387	0.411	0.391	0.396	0.026	0.415	0.386	0.393	0.398	0.030
14	0.423	0.446	0.433	0.434	0.023	0.409	0.403	0.402	0.405	0.008
16	0.434	0.456	0.444	0.445	0.022	0.419	0.398	0.409	0.409	0.021
17.5	0.448	0.471	0.439	0.453	0.033	0.418	0.398	0.409	0.408	0.020

100 μM UO ₂ ²⁺						150 μM UO ₂ ²⁺				
Time (h)	1	2	3	ave	95% CI	1	2	3	ave	95% CI
0	0.131	0.134	0.129	0.131	0.005	0.138	0.142	0.132	0.137	0.010
4	0.189	0.199	0.185	0.191	0.014	0.191	0.18	0.187	0.186	0.011
8	0.269	0.279	0.259	0.269	0.02	0.217	0.207	0.205	0.210	0.013
10	0.312	0.324	0.319	0.318	0.012	0.254	0.261	0.249	0.255	0.012
12	0.328	0.321	0.322	0.324	0.008	0.235	0.211	0.229	0.225	0.025
14	0.325	0.347	0.339	0.337	0.022	0.237	0.216	0.225	0.226	0.021
16	0.324	0.359	0.344	0.342	0.035	0.229	0.216	0.224	0.223	0.013
17.5	0.338	0.347	0.337	0.341	0.011	0.235	0.229	0.228	0.231	0.008

200 μM UO ₂ ²⁺					
Time (h)	1	2	3	ave	95% CI
0	0.137	0.147	0.141	0.142	0.010
4	0.173	0.161	0.159	0.164	0.015
8	0.19	0.177	0.173	0.18	0.018
10	0.219	0.215	0.218	0.217	0.004
12	0.205	0.189	0.199	0.198	0.016
14	0.209	0.189	0.197	0.198	0.020
16	0.217	0.193	0.189	0.200	0.030
17.5	0.217	0.201	0.187	0.202	0.030

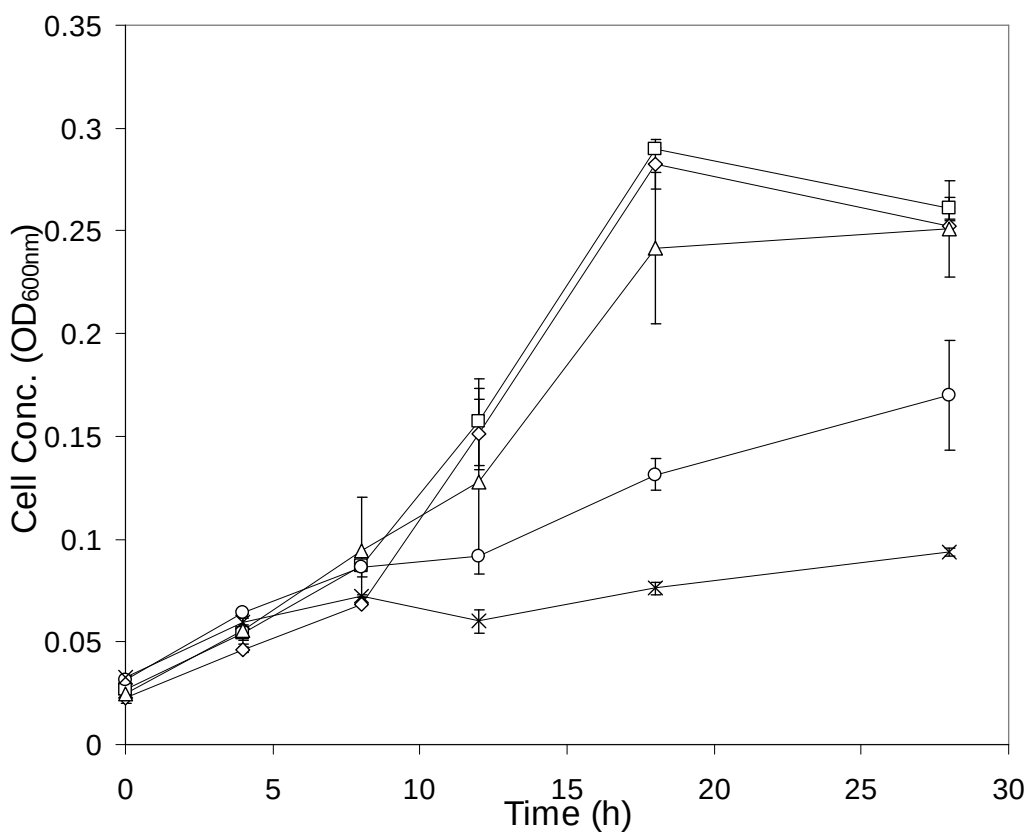


Figure 23. Growth curves of Isolate A growing on ethanol in the presence of UO_2^{2+} and excess carbonate (◇ = UO_2^{2+} free, □ = 50 μM UO_2^{2+} , Δ = 100 μM UO_2^{2+} , ○ = 150 μM UO_2^{2+} , and × = 200 μM UO_2^{2+}). Data points represent the average of triplicate measurements, and error bars correspond to 95% confidence intervals.

Table 11. OD_{600nm} values obtained during the growth of Isolate A on ethanol in the presence of excess carbonate and UO₂²⁺. Average values correspond to the average of triplicate measurements, and errors are reported as 95% confidence intervals.

Ethanol 0 μM UO ₂ ²⁺						50 μM UO ₂ ²⁺				
Time (h)	1	2	3	ave	95% CI	1	2	3	ave	95% CI
0	0.021	0.024	0.022	0.022	0.003	0.027	0.026	0.021	0.025	0.006
4	0.045	0.047	0.035	0.042	0.013	0.057	0.052	0.05	0.053	0.007
8	0.068	0.069	0.065	0.067	0.004	0.091	0.083	0.075	0.083	0.016
12	0.163	0.139	0.142	0.148	0.026	0.172	0.142	0.15	0.155	0.031
18	0.274	0.291	0.271	0.279	0.022	0.29	0.289	0.276	0.285	0.016
28	0.25	0.254	0.248	0.251	0.006	0.257	0.265	0.255	0.259	0.011

100 μM UO ₂ ²⁺						150 μM UO ₂ ²⁺				
Time (h)	1	2	3	ave	95% CI	1	2	3	ave	95% CI
0	0.028	0.028	0.028	0.028	0.000	0.03	0.033	0.042	0.035	0.012
4	0.06	0.059	0.054	0.058	0.006	0.065	0.064	0.058	0.062	0.008
8	0.101	0.075	0.089	0.088	0.026	0.086	0.086	0.075	0.082	0.013
12	0.163	0.089	0.121	0.124	0.045	0.092	0.092	0.088	0.091	0.005
18	0.256	0.159	0.18	0.198	0.037	0.126	0.137	0.121	0.128	0.016
28	0.262	0.247	0.27	0.260	0.023	0.151	0.189	0.161	0.167	0.039

200 μM UO ₂ ²⁺					
Time (h)	1	2	3	ave	95% CI
0	0.033	0.032	0.031	0.032	0.002
4	0.061	0.058	0.049	0.056	0.0125
8	0.072	0.073	0.068	0.071	0.0053
12	0.064	0.056	0.065	0.061	0.0014
18	0.078	0.074	0.069	0.073	0.0042
28	0.092	0.095	0.101	0.095	0.0014

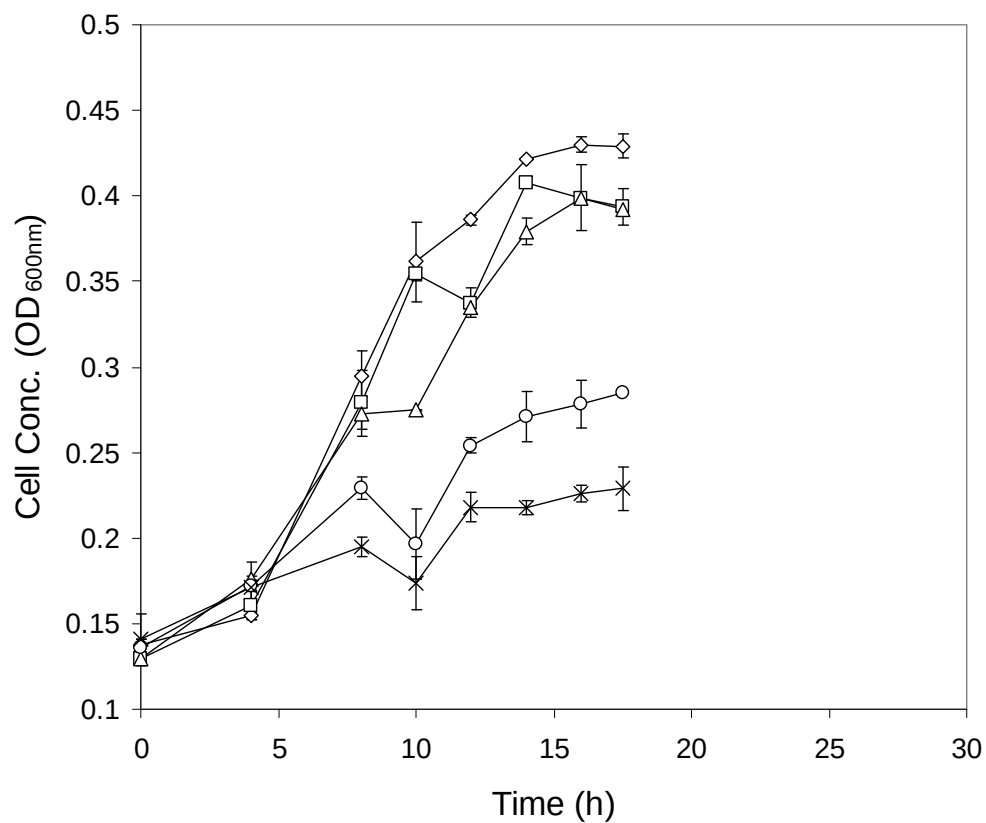


Figure 24. Growth curves of Isolate A growing on lactate in the presence of UO_2^{2+} and excess carbonate ($\diamond = \text{UO}_2^{2+}$ free, $\square = 50 \mu\text{M UO}_2^{2+}$, $\Delta = 100 \mu\text{M UO}_2^{2+}$, $\circ = 150 \mu\text{M UO}_2^{2+}$, and $\times = 200 \mu\text{M UO}_2^{2+}$). Data points represent the average of triplicate measurements, and error bars correspond to 95% confidence intervals.

Table 12. OD_{600nm} values obtained during the growth of Isolate A on lactate in the presence of excess carbonate and UO₂²⁺. Average values correspond to the average of triplicate measurements, and errors are reported as 95% confidence intervals.

Lactate	0 μM UO_2^{2+}					50 μM UO_2^{2+}				
Time (h)	1	2	3	ave	error	1	2	3	ave	error
0	0.14	0.136	0.129	0.135	0.011	0.138	0.135	0.129	0.134	0.009
4	0.156	0.153	0.152	0.154	0.004	0.163	0.175	0.16	0.166	0.016
8	0.305	0.284	0.292	0.294	0.021	0.307	0.28	0.279	0.289	0.032
10	0.345	0.378	0.355	0.359	0.034	0.375	0.37	0.369	0.371	0.006
12	0.388	0.384	0.388	0.387	0.005	0.349	0.361	0.339	0.350	0.022
14	0.421	0.422	0.419	0.421	0.003	0.427	0.431	0.421	0.426	0.010
16	0.427	0.433	0.425	0.428	0.008	0.406	0.433	0.399	0.413	0.036
17.5	0.424	0.434	0.425	0.428	0.011	0.407	0.422	0.398	0.409	0.024

100 μM UO_2^{2+}						150 μM UO_2^{2+}				
Time (h)	1	2	3	ave	error	1	2	3	ave	error
0	0.141	0.137	0.135	0.138	0.006	0.148	0.147	0.139	0.145	0.010
4	0.19	0.188	0.179	0.186	0.012	0.183	0.189	0.177	0.183	0.012
8	0.299	0.286	0.249	0.278	0.052	0.25	0.246	0.237	0.244	0.013
10	0.294	0.295	0.269	0.286	0.029	0.217	0.208	0.202	0.209	0.015
12	0.357	0.36	0.344	0.354	0.017	0.277	0.273	0.268	0.273	0.009
14	0.401	0.412	0.396	0.403	0.016	0.297	0.289	0.287	0.291	0.011
16	0.425	0.429	0.417	0.424	0.012	0.301	0.301	0.299	0.300	0.002
17.5	0.421	0.42	0.411	0.417	0.011	0.308	0.308	0.301	0.306	0.008

200 μM UO_2^{2+}					
Time (h)	1	2	3	ave	error
0	0.164	0.143	0.14	0.149	0.026
4	0.197	0.176	0.172	0.182	0.027
8	0.217	0.209	0.199	0.208	0.018
10	0.201	0.179	0.177	0.186	0.027
12	0.232	0.244	0.237	0.238	0.012
14	0.241	0.235	0.24	0.239	0.006
16	0.25	0.243	0.239	0.244	0.011
17.5	0.259	0.241	0.227	0.242	0.032

APPENDIX D

OPTICAL DENSITY VERSUS PROTEIN CONCENTRATION STANDARD CURVES

Standard curves were generated for both Isolate A and *Pseudomonas aeruginosa* PAO1 so that protein concentrations could be calculated using spectroscopic measurements. Protein concentrations were measured using the Bradford assay (1). Optical density was measured using a Thermo Electron Multiskan UV-Vis spectrophotometer and 48-well Corning plates. For Isolate A, optical density (OD) at 600 nm of a series of 1 mL liquid cell samples of six different cell concentrations was measured (Table 13). These cultures were then centrifuged (8,000 x g for 5 min) and washed three times with nano-pure water in low-protein binding microcentrifuge tubes (Axygen Scientific Inc., Union City, CA). The cell pellet was resuspended in 850 μ L of a 1N NaOH solution, transferred to glass culture tubes with metal caps, and placed in a 98° C oven for 10 min. All transfers were carried out with low-protein binding Axygen pipette tips. The solution was then neutralized with 140 μ L of a 6 N HCl solution. 500 μ L of the resulting solution was then mixed with an equal volume of Coomassie dye (Pierce Protein Research Products, Rockford, IL) and the OD at 595 nm was measured (Table 14). Protein concentrations were calculated using a standard curve generated using bovine serum albumin standards (Table 15 and Figure 25). Albumin standards were diluted with a 1 M NaCl solution prepared using nano-pure water. A linear relationship ($R^2 > 0.95$) between OD_{600nm} and protein concentration was then established (Figure 26).

An identical procedure was carried out with *Pseudomonas aeruginosa* PAO1 and generated similar results. Table 16 shows the initial OD_{600nm} measurements, Table 17 shows the corresponding protein concentrations based on a standard curve generated

using known protein standards (data not shown), and Figure 27 shows the resulting linear relationship ($R^2 > 0.98$) between OD_{600nm} and protein concentration.

Table 13. Optical density of liquid cultures of Isolate A of varying concentration. Average represents the average of triplicate measurements, error corresponds to the standard deviation of the triplicate measurements.

OD ₆₀₀ of liquid culture of Isolate A				
1	2	3	ave	Std Dev
0.102	0.099	0.099	0.100	0.0017
0.132	0.137	0.13	0.133	0.0036
0.157	0.161	0.154	0.157	0.0035
0.182	0.188	0.182	0.184	0.0035
0.229	0.241	0.224	0.231	0.0087
0.486	0.47	0.447	0.468	0.0196

Table 14. OD_{595nm} of lysed Isolate A cultures exposed to Coomassie dye. This absorbance was then converted to protein concentrations using the standard curve generated using bovine serum albumin standards (Figure 1). Average represents the average of triplicate measurements, error corresponds to the standard deviation of the triplicate measurements.

OD _{595nm} of lysed cells exposed to Coomassie dye			Isolate A protein concentration according to standard curve (Figure 1)				
1	2	3	1	2	3	ave	Std Dev
0.406	0.395	0.455	1.67	1.17	3.90	2.24	1.45
0.471	0.441	0.484	4.62	3.26	5.21	4.37	1.00
0.51	0.507	0.482	6.40	6.26	5.12	5.93	0.70
0.539	0.577	0.552	7.71	9.44	8.30	8.49	0.88
0.662	0.754	0.676	13.30	17.49	13.94	14.91	2.25
0.922	0.899	0.928	25.12	24.08	25.40	24.87	0.70

Table 15. OD_{595nm} of known protein concentrations using bovine serum albumin standards. This data was used to generate a standard curve relating OD_{595nm} to protein concentration (Figure 1). Average represents the average of triplicate measurements, error corresponds to the standard deviation of the triplicate measurements.

mg/L protein from bovine serum albumin standard	OD _{595nm}	average	Std Dev
0.0	0.409		
0.0	0.409		
0.0	0.39	0.403	0.011
2.5	0.522		
2.5	0.497		
2.5	0.502	0.507	0.013
5.0	0.592		
5.0	0.566		
5.0	0.598	0.585	0.017
10	0.695		
10	0.705		
10	0.71	0.703	0.008
15	0.775		
15	0.821		
15	0.808	0.801	0.024
20	0.906		
20	0.935		
20	0.938	0.926	0.018

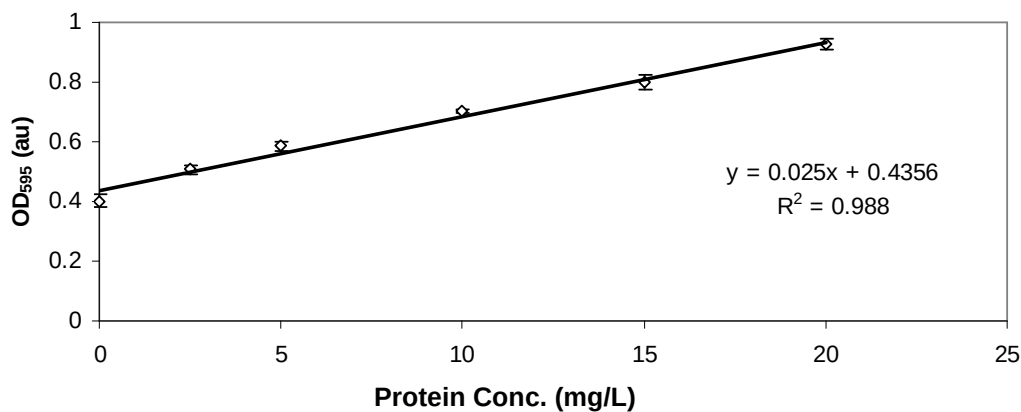


Figure 25. OD_{595nm} vs protein concentration standard curve generated from data presented in Table 2. The embedded equation was used to convert the OD_{595nm} values in Table 14 to protein concentrations. Data points represent the average of triplicate measurements, error bars corresponds to the standard deviation of the triplicate measurements.

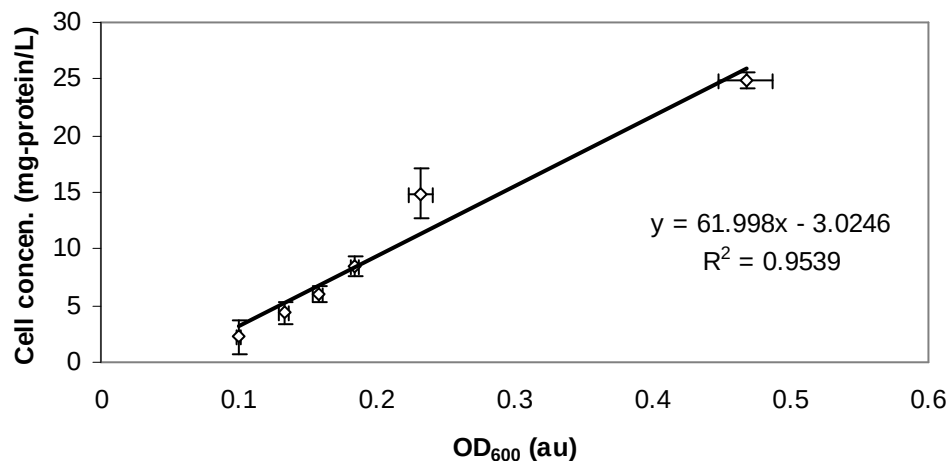


Figure 26. Linear relationship between OD_{600nm} measurements and corresponding protein concentrations, generated using data found in Tables 1 and 2. Data points represent the average of triplicate measurements, error bars corresponds to the standard deviation of the triplicate measurements.

Table 16. Optical density of liquid cultures of PAO1 of varying concentration. Average represents the average of triplicate measurements, error corresponds to the standard deviation of the triplicate measurements.

OD ₆₀₀ of liquid cultures of PAO1				
1	2	3	ave	Std Dev
0.087	0.087	0.087	0.087	1.7E-17
0.262	0.268	0.265	0.265	3.0E-03
0.449	0.443	0.425	0.439	1.2E-02
0.615	0.595	0.584	0.598	1.6E-02

Table 17. OD_{595nm} of lysed PAO1 cultures exposed to Coomassie dye. This absorbance was then converted to protein concentrations using the standard curve generated using bovine serum albumin standards. Average represents the average of triplicate measurements, error corresponds to the standard deviation of the triplicate measurements.

OD _{595nm} of lysed cells exposed to Coomassie dye			PAO1 protein concentration according to standard curve				
1	2	3	1	2	3	ave	Std Dev
0.291	0.294	0.296	0.0696203	0.278481	0.4177215	0.2552743	1.8E-01
0.555	0.55	0.534	18.449367	18.101266	16.987342	17.845992	7.6E-01
0.758	0.71	0.708	32.582278	29.240506	29.101266	30.308017	2.0E+00
0.857	0.847	0.859	39.474684	38.778481	39.613924	39.28903	4.5E-01

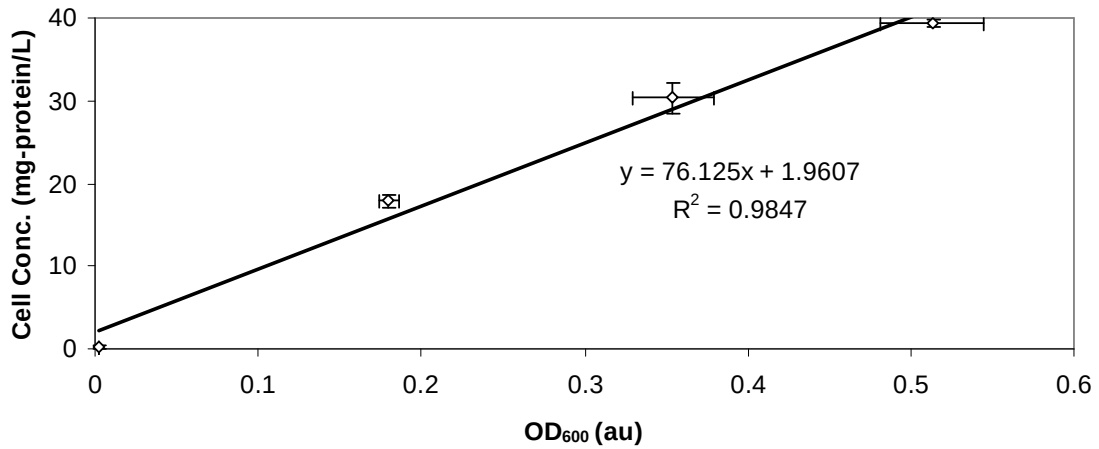


Figure 27. Linear relationship between PAO1 OD_{600nm} measurements and corresponding protein concentrations, generated using data found in Tables 4 and 5. Data points represent the average of triplicate measurements, error bars corresponds to the standard deviation of the triplicate measurements.

References

1. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254.

APPENDIX E

ACCUMULATION OF UO_2^{2+} BY ISOLATE A AS A FUNCTION OF TIME

In Chapter 2, it was stated that within 10 minutes, equilibrium between aqueous UO_2^{2+} and UO_2^{2+} accumulated within cells of Isolate A is reached. In this appendix, data supporting this statement is presented. Media composition and experimental conditions and procedures can be found in Chapter 2. In this experiment, only lactate was used as the growth substrate. UO_2^{2+} was added to a concentration of 50 μM , and the cell concentration was 22.5 mg-protein/L. Immediately upon exposure to UO_2^{2+} , 2 mL of aqueous samples of the culture were removed at 30 sec intervals to produce 1 mL filtered (0.2 μm) samples. Cell associated UO_2^{2+} was defined as the difference in UO_2^{2+} concentration between the UO_2^{2+} initially present, and the UO_2^{2+} recovered in the filtered samples. UO_2^{2+} was quantified using a KPA, and cells in unfiltered samples were digested (exposure to 8 N HNO_3 for 1 hr) prior to KPA analysis as described in Chapter 2. Table 18 shows the UO_2^{2+} concentration in the filtered samples with time. Table 19 shows the amount of cell associated UO_2^{2+} , and Table 20 shows the amount of UO_2^{2+} accumulated per mg of cell protein. Data in Table 20 is graphed in Figure 28. Experiments were performed in duplicate, and average values correspond to the average of duplicate measurements. Reported errors and error bars correspond to 95% confidence intervals.

Table 18. Non-cell associated aqueous UO_2^{2+} recovered in filtered (0.2 μm) samples. Concentrations are reported in μM . Reported errors correspond to 95% confidence intervals of duplicate measurements.

UO_2^{2+} in 0.2 μm filtered samples				
Time (min)	1	2	ave	95 % CI
0	47.155	41.144	44.150	8.501
0.5	42.303	41.172	41.738	1.599
1	39.639	39.627	39.633	0.017
1.5	40.337	38.646	39.492	2.391
2	39.275	37.518	38.397	2.485
2.5	38.57	38.828	38.699	0.365
3	40.209	38.637	39.423	2.223
3.5	37.685	36.967	37.326	1.015
4	37.856	37.121	37.489	1.039
4.5	37.588	39.692	38.640	2.976
5	39.355	37.204	38.280	3.042

Table 19. Cell associated aqueous UO_2^{2+} concentrations. Concentrations are reported in μM . Reported errors correspond to 95% confidence intervals of duplicate measurements.

cell associated UO_2^{2+} (50 μM - UO_2^{2+} in 0.2 μm filtered samples)				
Time (min)	1	2	ave	95% CI
0	2.845	8.856	5.851	8.501
0.5	7.697	8.828	8.263	1.599
1	10.361	10.373	10.367	0.017
1.5	9.663	11.354	10.509	2.391
2	10.725	12.482	11.604	2.485
2.5	11.43	11.172	11.301	0.365
3	9.791	11.363	10.577	2.223
3.5	12.315	13.033	12.674	1.015
4	12.144	12.879	12.512	1.039
4.5	12.412	10.308	11.360	2.976
5	10.645	12.796	11.721	3.042

Table 20. Cell associated aqueous UO_2^{2+} concentrations on a per mg-cell protein basis. Concentrations are reported in μM . Reported errors correspond to 95% confidence intervals of duplicate measurements.

UO_2^{2+} accumulated ($\mu\text{M}/\text{mg-protein}$)				
Time (min)	1	2	ave	95% CI
0.0	0.126	0.393	0.260	0.377
0.5	0.342	0.392	0.367	0.071
1.0	0.460	0.461	0.460	0.001
1.5	0.429	0.504	0.467	0.106
2.0	0.476	0.554	0.515	0.110
2.5	0.508	0.496	0.502	0.016
3.0	0.435	0.505	0.470	0.099
3.5	0.547	0.579	0.563	0.045
4.0	0.539	0.572	0.556	0.046
4.5	0.551	0.458	0.504	0.132
5.0	0.473	0.568	0.520	0.135

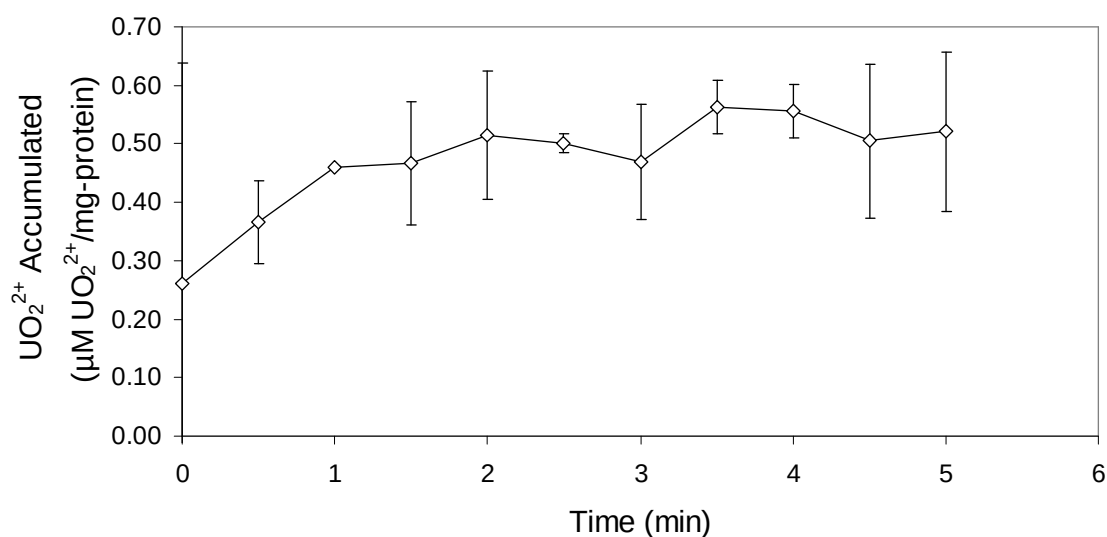


Figure 28. UO_2^{2+} accumulation within Isolate A cells metabolizing lactate. After 5 minutes, the amount of UO_2^{2+} accumulated does not change significantly, indicating that equilibrium is quickly reached. Error bars correspond to 95% confidence intervals of duplicate measurements.

APPENDIX F

BIOACCUMULATION OF UO_2^{2+} BY ISOLATE A

This appendix contains tabulated UO_2^{2+} concentration (μM) measurements used to generate Figure 7 of Chapter 2. Media composition and experimental conditions and procedures can be found in Chapter 2. In each treatment, UO_2^{2+} was added to a concentration of $50 \mu\text{M}$. UO_2^{2+} was quantified using a KPA, and cells in unfiltered samples were digested prior to KPA analysis as described in Chapter 2. Cells were washed five times in $\text{pH} = 5.5$, $100 \mu\text{M}$ EDTA solution, as described in Chapter 2. Tables 21 and 22 contain the data from the low and high bicarbonate systems, respectively, as defined in Chapter 2. The data presented here also substantiates the claim made in Chapter 2 that accumulated UO_2^{2+} is not loosely bound, as the UO_2^{2+} mass balance is closed between the filtered ($0.2 \mu\text{m}$) samples and the washed cells. Experiments were performed in triplicate, and average values correspond to the average of triplicate measurements. Reported errors correspond to 95% confidence intervals.

Table 21. UO_2^{2+} bioaccumulation by Isolate A during growth on one of four substrates in low bicarbonate media. Concentrations are reported in $\mu\text{M UO}_2^{2+}$. Average values correspond to the average of triplicate measurements. Reported errors correspond to 95% confidence intervals of triplicate measurements, including cell concentration.

Butyrate	1	2	3	average	95% CI	rel 95% CI
unfiltered samples	47.963	47.605	47.207	47.592	0.756	0.016
washed cells	31.994	34.136	32.988	33.039	2.144	0.065
filtered samples	20.389	21.013	20.755	20.719	0.627	0.030
washed + filtered	52.383	55.149	53.743	53.758	2.771	0.052
cell concn (mg-protein/L)	95% CI	rel 95% CI	$\mu\text{M UO}_2^{2+}$ acc/mg-protein	95% CI		
74.47	0.47	0.01	0.44	0.029		

Dextrose	1	2	3	average	95% CI	rel 95% CI
unfiltered samples	49.596	49.924	50.112	49.877	0.522	0.010
washed cells	41.601	46.522	43.801	43.975	4.930	0.112
filtered samples	12.132	11.197	11.001	11.443	1.209	0.106
washed + filtered	53.733	57.719	54.802	55.418	6.139	0.111
cell concn (mg-protein/L)	95% CI	rel 95% CI	$\mu\text{M UO}_2^{2+}$ acc/mg-protein	95% CI		
78.93	0.14	0.002	0.56	0.062		

Ethanol	1	2	3	average	95% CI	rel 95% CI
unfiltered samples	50.333	47.871	48.996	49.067	2.465	0.050
washed cells	32.084	30.5	30.779	31.121	1.691	0.054
filtered samples	18.612	20.179	19.333	19.375	1.569	0.081
washed + filtered	50.696	50.679	50.112	50.496	3.260	0.065
cell concn (mg-protein/L)	95% CI	rel 95% CI	$\mu\text{M UO}_2^{2+}$ acc/mg-protein	95% CI		
77.28	1.43	0.02	0.40	0.023		

Lactate	1	2	3	average	95% CI	rel 95% CI
unfiltered samples	44.881	49.135	48.552	47.523	4.612	0.097
washed cells	27.873	28.817	26.919	27.870	1.898	0.068
filtered samples	22.605	21.458	23.001	22.355	1.603	0.072
washed + filtered	50.478	50.275	49.92	50.224	3.501	0.070

cell concn (mg- protein/L)	95% CI	rel 95% CI	$\mu\text{M UO}_2^{2+}$ acc/mg- protein	95% CI
80.83	0.56	0.01	0.35	0.024

Carbon free	1	2	3	average	95% CI	rel 95% CI
unfiltered samples	51.495	48.51	46.223	48.743	5.287	0.108
washed cells	15.46	12.251	13.235	13.649	3.288	0.241
filtered samples	33.129	34.88	32.404	33.471	2.546	0.076
washed + filtered	48.589	47.131	45.639	47.120	5.834	0.124

cell concn (mg- protein/L)	95% CI	rel 95% CI	$\mu\text{M UO}_2^{2+}$ acc/mg- protein	95% CI
80.67	0.90	0.01	0.17	0.041

Heat killed cell	1	2	3	average	95% CI	rel 95% CI
unfiltered samples	49.933	48.019	46.774	48.242	3.183	0.066
washed cells	11.84	13.726	11.799	12.455	2.202	0.177
filtered samples	40.034	35.512	37.709	37.752	4.523	0.120
washed + filtered	51.874	49.238	49.508	50.207	6.724	0.134

cell concn (mg- protein/L)	95% CI	rel 95% CI	$\mu\text{M UO}_2^{2+}$ acc/mg- protein	95% CI
80.01	1.22	0.02	0.16	0.03

Table 22. UO_2^{2+} bioaccumulation by Isolate A during growth on one of four substrates in high bicarbonate media. Concentrations are reported in $\mu\text{M UO}_2^{2+}$. Average values correspond to the average of triplicate measurements. Reported errors correspond to 95% confidence intervals of triplicate measurements.

Butyrate	1	2	3	average	95% CI	rel 95% CI
unfiltered samples	48.771	49.412	53.578	50.587	2.610	0.052
washed cells	5.887	6.543	6.367	6.266	0.340	0.054
filtered samples	43.609	47.378	41.649	44.212	2.912	0.066
washed + filtered	49.496	53.921	48.016	50.478	3.072	0.061
cell concn (mg-protein/L)	95% CI	rel 95% CI	$\mu\text{M UO}_2^{2+}$ acc/mg-protein	95% CI		
80.61	0.39	0.0049	0.078	0.009		
Dextrose	1	2	3	average	95% CI	rel 95% CI
unfiltered samples	50.327	46.002	47.578	47.969	2.189	0.046
washed cells	9.276	9.321	9.322	9.306	0.026	0.003
filtered samples	39.558	36.112	39.227	38.299	1.901	0.050
washed + filtered	48.834	45.433	48.549	47.605	1.887	0.040
cell concn (mg-protein/L)	95% CI	rel 95% CI	$\mu\text{M UO}_2^{2+}$ acc/mg-protein	95% CI		
80.11	0.03	0.0003	0.12	0.003		
Ethanol	1	2	3	average	95% CI	rel 95% CI
unfiltered samples	49.556	51.009	46.114	48.893	2.514	0.051
washed cells	7.043	7.09	7.006	7.046	0.042	0.006
filtered samples	40.128	49.919	46.276	45.441	4.949	0.109
washed + filtered	47.171	57.009	53.282	52.487	4.967	0.095
cell concn (mg-protein/L)	95% CI	rel 95% CI	$\mu\text{M UO}_2^{2+}$ acc/mg-protein	95% CI		
79.02	0.05	0.0007	0.088	0.003		

Lactate	1	2	3	average	95% CI	rel 95% CI
unfiltered samples	48.454	44.021	48.321	46.932	2.522	0.054
washed cells	4.91	5.401	5.416	5.242	0.288	0.055
filtered samples	43.003	40.467	43.223	42.231	1.532	0.036
washed + filtered	47.913	45.868	48.639	47.473	1.437	0.030

cell concn (mg-protein/L)	95% CI	rel 95% CI	$\mu\text{M UO}_2^{2+}$ acc/mg-protein	95% CI
81.03	0.18	0.0022	0.066	0.006

Carbon free	1	2	3	average	95% CI	rel 95% CI
unfiltered samples	51.442	46.349	49.236	49.009	2.554	0.052
washed cells	4.019	5.013	4.04	4.357	0.568	0.130
filtered samples	44.439	40.248	42.549	42.412	2.099	0.049
washed + filtered	48.458	45.261	46.589	46.769	1.606	0.034

cell concn (mg-protein/L)	95% CI	rel 95% CI	$\mu\text{M UO}_2^{2+}$ acc/mg-protein	95% CI
80.55	1.07	0.0133	0.054	0.011

Heat killed cell	1	2	3	average	95% CI	rel 95% CI
unfiltered samples	44.239	49.253	48.222	47.238	2.648	0.056
washed cells	5.012	5.185	4.605	4.934	0.298	0.060
filtered samples	41.952	43.955	40.993	42.300	1.511	0.036
washed + filtered	46.964	49.14	45.598	47.234	1.786	0.038

cell concn (mg-protein/L)	95% CI	rel 95% CI	$\mu\text{M UO}_2^{2+}$ acc/mg-protein	95% CI
80.37	0.42	0.0053	0.062	0.007

APPENDIX G

TRANSMISSION ELECTRON MICROSCOPY IMAGES OF ISOLATE A

Transmission electron microscopy images of Isolate A were obtained, and include images of uranium (U) exposed cells and U-free cells. Images were taken to visualize UO_2^{2+} accumulation within cells of Isolate A to better understand the location of the accumulated UO_2^{2+} , and to compare the results with previously published results.

Liquid cultures were grown aerobically at 20°C in 50 mL of sterile (30 min at 121°C) simulated groundwater media (SGM) as described in Chapter 3. Dextrose (Fisher Scientific, Fair Lawn, NJ) was added to a concentration of 2.5 mM and served as sole carbon source. Cells were grown in 250 mL baffled shaker flasks rotating at 100 rpm. Upon reaching mid-exponential growth phase, cells were pelleted ($6,000 \times g$ for 20 min) and washed three times in fresh media. Washed cells were resuspended fresh media to a cell concentration of 20 mg-cell protein/L, and allowed to sit at 20°C for 1 hr. The culture was then divided. Into one of the two cultures UO_2^{2+} was added (UO_2Cl_2 , International Bio-Analytical Industries Inc., Boca Raton, Florida) to a concentration of 50 μM , and allowed to equilibrate for an additional 15 min. The other culture was later stained with OsO_4 . The U-exposed cultures were then each washed five times in a 100 μM ethylenediamine triacetic acid (EDTA) solution at pH = 5.5 to remove any loosely bound UO_2^{2+} from the cell surface, as described in Chapter 3.

The final cultures were pelleted again and resuspended in 50% glutaraldehyde (Fisher Scientific, Fair Lawn, NJ) and fixed overnight at 4°C. Cells were then dehydrated sequentially using 30%, 50%, 70%, 90%, and 100% ethanol solutions (Sigma-Aldrich, St. Louis, MO). Cells were washed two final times in 100% ethanol to remove any remaining moisture. U-free cells were stained with OsO_4 . U-exposed cells were not

stained, since the accumulated UO_2^{2+} provided enough electron density to stain the cells without the addition of OsO_4 .

After the final ethanol wash, cells were resuspended in Spurrs resin and placed in a 60°C oven. overnight. Cells were thin-sectioned using a Reichert ultramicrotome. Images were taken using a Proscan 2048x2048 – CCD camera connected to a Zeiss LEO 912 TEM at the MSU TEM facility.

Figure 29 shows the image of a U-free, OsO_4 stained Isolate A cell, revealing a short rod approximately 1 μm wide and 2 μm long, consistent with FSEM images included in Chapter 5. Figure 30 shows another OsO_4 stained, U-free cell.

Figures 31 and 32 are TEM images of UO_2^{2+} exposed cells. Since no stain was used as part of this cell preparation, any indication of high electron density is due to the presence of accumulated UO_2^{2+} . Consistent with previous studies with UO_2^{2+} exposed *Pseudomonas* sp. cells, significant UO_2^{2+} accumulation can be observed within the cells, particularly in the cytoplasm (1). Future work could employ the use of high-resolution TEM to further investigate UO_2^{2+} accumulation in A, as this would have implications regarding how UO_2^{2+} bioaccumulation in Isolate A affects UO_2^{2+} fate and mobility.

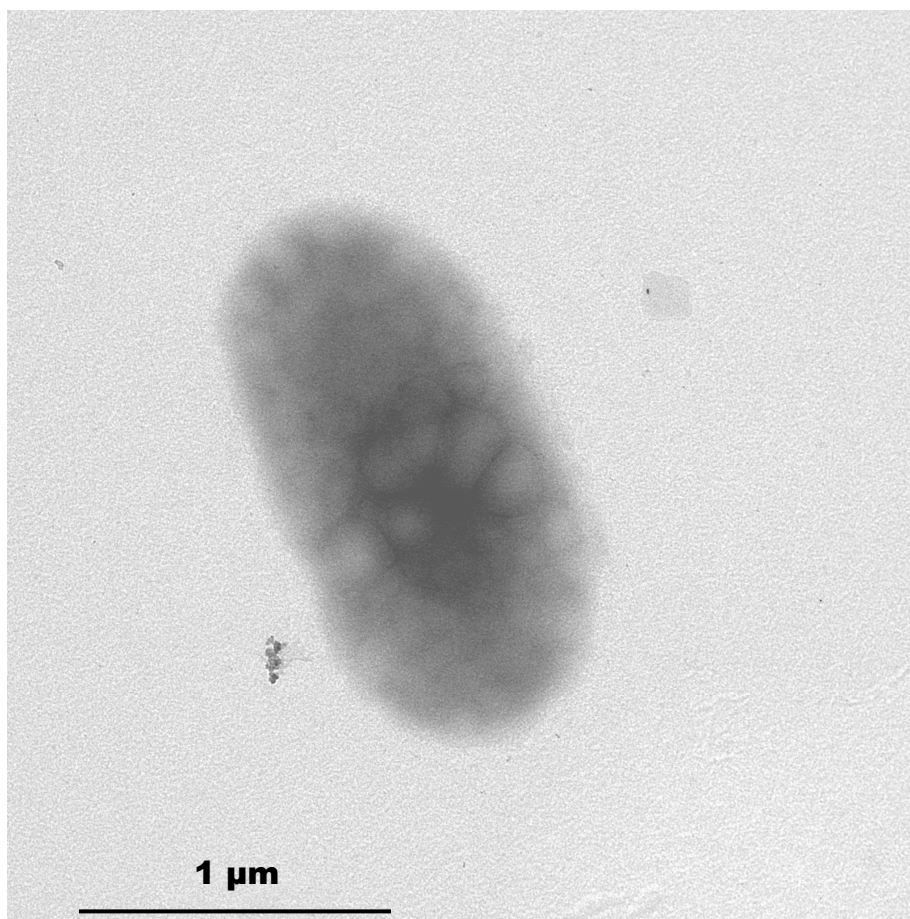


Figure 29. TEM image of OsO_4 stained Isolate A cell not exposed to UO_2^{2+} .

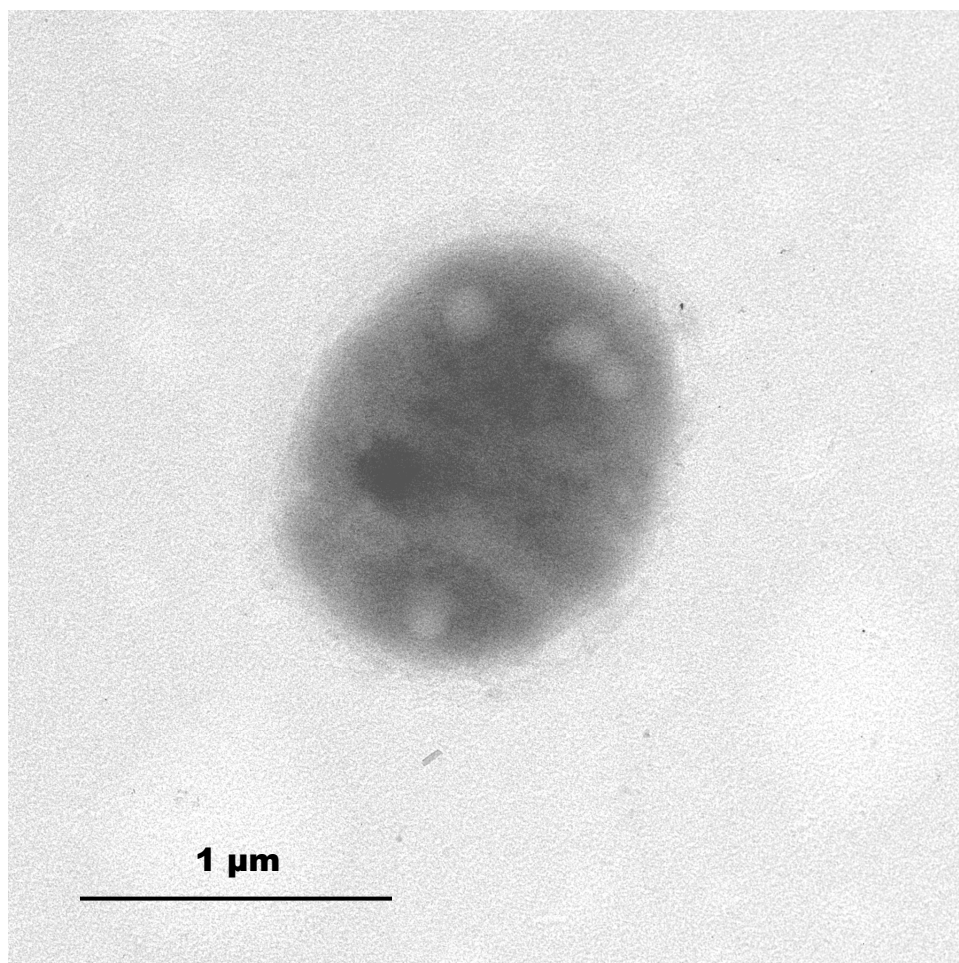


Figure 30. TEM image of OsO_4 stained Isolate A cell not exposed to UO_2^{2+} .

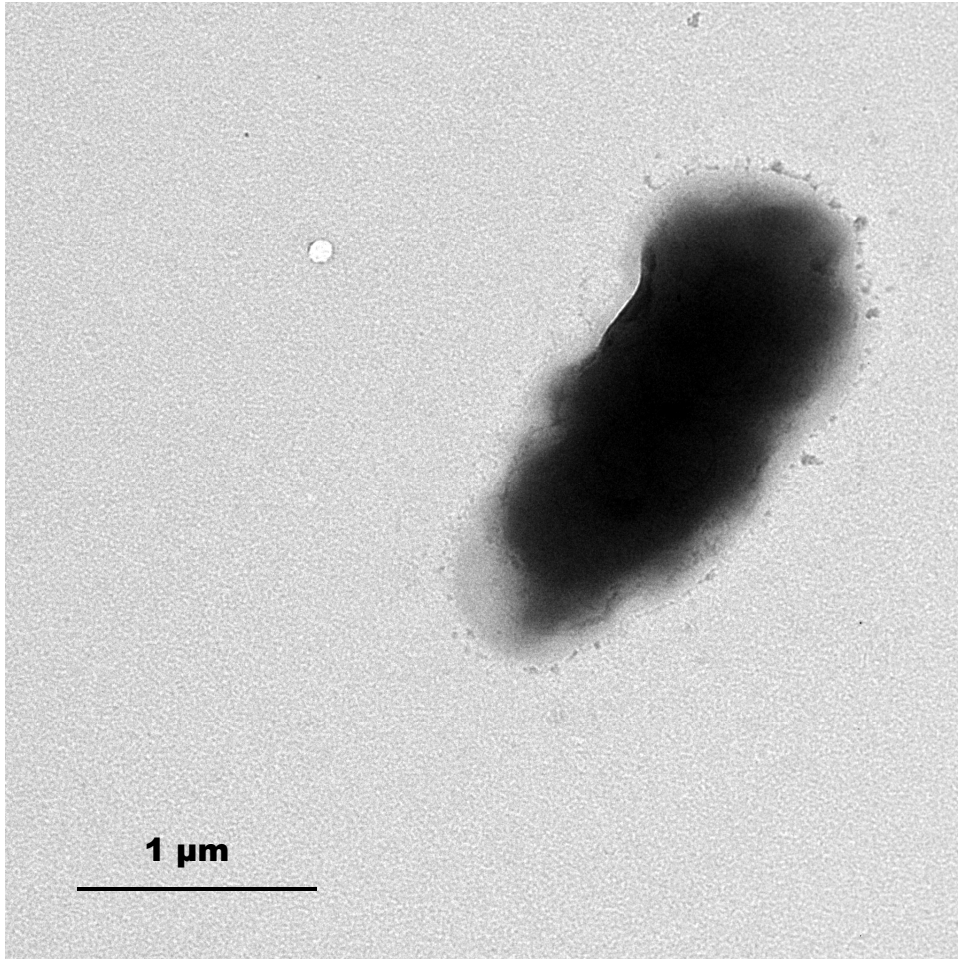


Figure 31. TEM image of unstained Isolate A cell after exposure to UO_2^{2+} .

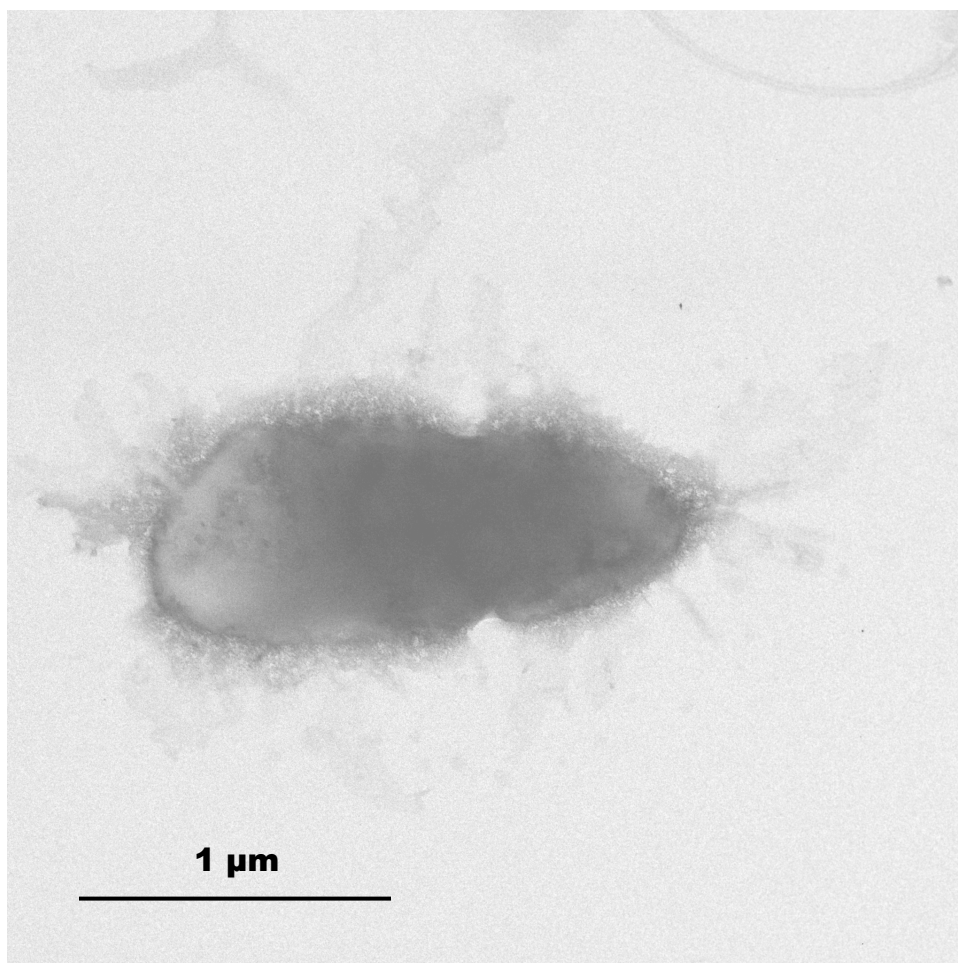


Figure 32. TEM image of unstained Isolate A cell after exposure to UO_2^{2+} .

References

1. Strandberg GW, Shumate II, Starling E, Parrott Jr JR. 1981. Microbial Cells as Biosorbents for Heavy Metals: Accumulation of Uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa*. *Appl Environ Microbiol.*41:237-245.

APPENDIX H

PSEUDOMONAS AERUGINOSA PAO1 AND *METHYLOBACTERIUM EXTORQUENS*
AM1 GROWTH DATA

In this appendix, the raw OD_{600nm} measurements used to generate Figure 11(a-d) in Chapter 4 are tabulated. Growth conditions and experimental procedures are described in Chapter 4. Tables 23 and 24 contain growth data of PAO1 on ethanol and dextrose, respectively, over a range of UO₂²⁺ concentrations. Table 25 and 26 contain growth data of AM1 on methanol and methylamine, respectively, over a range of UO₂²⁺ concentrations. Experiments were performed in triplicate, and average values correspond to the average of triplicate measurements. Reported errors correspond to 95% confidence intervals.

Table 23. OD_{600nm} measurements used to describe the growth of PAO1 on ethanol in the presence of UO₂²⁺. Experiments were performed in triplicate, and average values correspond to the average of triplicate measurements. Reported errors correspond to 95% confidence intervals.

Ethanol	0 μM UO_2^{2+}				
Time (h)	1	2	3	ave	95% CI
0	0.099	0.092	0.095	0.095	0.007
18	0.093	0.1	0.098	0.097	0.007
24	0.098	0.096	0.092	0.095	0.006
29	0.12	0.136	0.125	0.127	0.016
32	0.161	0.167	0.166	0.165	0.006
35	0.182	0.18	0.183	0.182	0.003
38	0.203	0.235	0.224	0.221	0.033
41	0.205	0.213	0.21	0.209	0.008
47	0.218	0.227	0.22	0.222	0.009
51	0.252	0.252	0.255	0.253	0.003

0.5 μM UO_2^{2+}					
Time (h)	1	2	3	ave	95% CI
0	0.093	0.094	0.097	0.095	0.004
18	0.096	0.087	0.097	0.093	0.011
24	0.103	0.104	0.106	0.104	0.003
29	0.102	0.1	0.101	0.101	0.002
32	0.102	0.102	0.104	0.103	0.002
35	0.123	0.147	0.135	0.135	0.024
38	0.15	0.174	0.166	0.163	0.024
41	0.211	0.192	0.205	0.203	0.019
47	0.222	0.231	0.225	0.226	0.009
51	0.24	0.253	0.248	0.247	0.013

1.0 μM UO_2^{2+}					
Time (h)	1	2	3	ave	95% CI
0	0.094	0.096	0.094	0.095	0.002
18	0.086	0.089	0.091	0.089	0.005
24	0.086	0.087	0.092	0.088	0.006
29	0.089	0.091	0.089	0.090	0.002
32	0.085	0.09	0.088	0.088	0.005
35	0.087	0.099	0.1	0.095	0.014
38	0.09	0.114	0.106	0.103	0.024
41	0.1	0.09	0.102	0.097	0.013
47	0.161	0.099	0.122	0.127	0.063
51	0.205	0.153	0.166	0.175	0.054

Table 24. OD_{600nm} measurements used to describe the growth of PAO1 on dextrose in the presence of UO₂²⁺. Experiments were performed in triplicate, and average values correspond to the average of triplicate measurements. Reported errors correspond to 95% confidence intervals.

Dextrose	0 μM UO_2^{2+}				
Time (h)	1	2	3	ave	95% CI
0	0.102	0.101	0.1	0.101	0.002
5	0.124	0.12	0.119	0.121	0.005
10	0.167	0.162	0.163	0.164	0.005
16.5	0.354	0.368	0.355	0.359	0.016
22.5	0.372	0.37	0.377	0.373	0.007
26	0.387	0.387	0.383	0.386	0.005

25 μM UO_2^{2+}					
Time (h)	1	2	3	ave	95% CI
0	0.105	0.104	0.104	0.104	0.001
5	0.125	0.127	0.127	0.126	0.002
10	0.165	0.17	0.165	0.167	0.006
16.5	0.324	0.349	0.352	0.342	0.031
22.5	0.387	0.389	0.374	0.383	0.016
26	0.397	0.4	0.403	0.400	0.006

100 μM UO_2^{2+}					
Time (h)	1	2	3	ave	95% CI
0	0.117	0.117	0.115	0.116	0.002
5	0.135	0.135	0.136	0.135	0.001
10	0.18	0.186	0.189	0.185	0.009
16.5	0.236	0.246	0.265	0.249	0.029
22.5	0.278	0.283	0.319	0.293	0.045
26	0.284	0.295	0.311	0.297	0.027

250 μM UO_2^{2+}					
Time (h)	1	2	3	ave	95% CI
0	0.138	0.135	0.136	0.136	0.003
5	0.134	0.129	0.13	0.131	0.005
10	0.138	0.126	0.13	0.131	0.012
16.5	0.13	0.122	0.127	0.126	0.008
22.5	0.129	0.123	0.124	0.125	0.006
26	0.127	0.114	0.125	0.122	0.014

Table 25. OD_{600nm} measurements used to describe the growth of AM1 on methanol in the presence of UO₂²⁺. Experiments were performed in triplicate, and average values correspond to the average of triplicate measurements. Reported errors correspond to 95% confidence intervals.

Methanol	0 μM UO_2^{2+}				
Time (h)	1	2	3	ave	95% CI
0	0.09	0.086	0.086	0.087	0.002
6	0.088	0.09	0.086	0.088	0.002
20	0.158	0.171	0.166	0.165	0.007
28	0.432	0.328	0.413	0.391	0.055
48	0.49	0.467	0.477	0.479	0.016

0.5 μM UO_2^{2+}					
Time (h)	1	2	3	ave	95% CI
0	0.086	0.097	0.085	0.089	0.007
6	0.085	0.088	0.086	0.086	0.002
20	0.205	0.179	0.161	0.182	0.022
28	0.316	0.338	0.318	0.324	0.012
48	0.322	0.344	0.38	0.351	0.041

1.0 μM UO_2^{2+}					
Time (h)	1	2	3	ave	95% CI
0	0.086	0.088	0.086	0.087	0.001
6	0.088	0.09	0.086	0.088	0.002
20	0.171	0.2	0.194	0.188	0.015
28	0.262	0.339	0.226	0.276	0.058
48	0.422	0.388	0.324	0.373	0.069

2.0 μM UO_2^{2+}					
Time (h)	1	2	3	ave	95% CI
0	0.086	0.094	0.085	0.088	0.005
6	0.085	0.088	0.086	0.086	0.002
20	0.168	0.199	0.192	0.186	0.016
28	0.278	0.29	0.242	0.270	0.025
48	0.286	0.31	0.325	0.306	0.028

Table 26. OD_{600nm} measurements used to describe the growth of AM1 on methylamine in the presence of UO₂²⁺. Experiments were performed in triplicate, and average values correspond to the average of triplicate measurements. Reported errors correspond to 95% confidence intervals.

Methylamine 0 μM UO ₂ ²⁺					
Time (h)	1	2	3	ave	95% CI
0	0.1	0.098	0.089	0.096	0.006
10	0.12	0.123	0.119	0.121	0.002
20	0.159	0.16	0.162	0.160	0.002
24	0.237	0.235	0.26	0.244	0.014
28	0.325	0.302	0.345	0.324	0.022
32	0.347	0.409	0.399	0.385	0.033
50	0.47	0.387	0.379	0.412	0.050

50 μM UO ₂ ²⁺					
Time (h)	1	2	3	ave	95% CI
0	0.088	0.095	0.096	0.093	0.004
10	0.098	0.112	0.1	0.103	0.008
20	0.111	0.129	0.113	0.118	0.010
24	0.121	0.125	0.129	0.125	0.004
28	0.16	0.182	0.167	0.170	0.011
32	0.138	0.179	0.159	0.159	0.021
50	0.289	0.324	0.328	0.314	0.021

125 μM UO ₂ ²⁺					
Time (h)	1	2	3	ave	95% CI
0	0.101	0.099	0.099	0.100	0.001
10	0.107	0.107	0.104	0.106	0.002
20	0.118	0.15	0.116	0.128	0.019
24	0.145	0.139	0.125	0.136	0.010
28	0.14	0.19	0.136	0.155	0.030
32	0.166	0.122	0.154	0.147	0.023
50	0.239	0.241	0.258	0.246	0.010

250 μM UO ₂ ²⁺					
Time (h)	1	2	3	ave	95% CI
0	0.093	0.089	0.091	0.091	0.002
10	0.099	0.088	0.097	0.095	0.006
20	0.107	0.113	0.109	0.110	0.003
24	0.122	0.121	0.115	0.119	0.004
28	0.105	0.112	0.114	0.110	0.005
32	0.115	0.11	0.109	0.111	0.003
50	0.116	0.109	0.113	0.113	0.004

APPENDIX I

UV-VIS ABSORBANCE DATA OF THE PQQ, PQQ + Ca^{2+} , PQQ + UO_2^{2+} , AND PQQ
+ Ca^{2+} + UO_2^{2+} SYSTEMS

This appendix contains the raw UV-Vis absorbance data of the PQQ, PQQ + Ca^{2+} , PQQ + UO_2^{2+} , and PQQ + Ca^{2+} + UO_2^{2+} systems used to generate Figure 12a in Chapter 4. PQQ and metals were each added to a concentration of 1 mM. Absorbance was measured between 400 and 500 nm at 1 nm increments using a Thermo Electron Multiskan Spectrum UV-Vis spectrophotometer as described in Chapter 4. Table 1 contains each absorbance measurement, in arbitrary units, for each combination.

Table 27. Absorbance values (au) of PQQ and PQQ bound to either Ca^{2+} or UO_2^{2+} . Data was used to generate Figure 3a of Chapter 4.

Absorbance (au)				
Wavelength (nm)	PQQ	PQQ + Ca^{2+}	PQQ + UO_2^{2+}	PQQ + Ca^{2+} + UO_2^{2+}
400	0.848	0.799	2.605	2.615
401	0.794	0.749	2.530	2.557
402	0.756	0.715	2.515	2.525
403	0.707	0.671	2.446	2.457
404	0.667	0.633	2.363	2.393
405	0.636	0.605	2.284	2.308
406	0.598	0.570	2.168	2.186
407	0.564	0.540	2.049	2.080
408	0.531	0.509	1.923	1.951
409	0.508	0.487	1.830	1.856
410	0.484	0.464	1.720	1.753
411	0.460	0.443	1.604	1.635
412	0.445	0.428	1.516	1.545
413	0.425	0.409	1.404	1.435
414	0.408	0.394	1.297	1.329
415	0.394	0.380	1.196	1.226
416	0.384	0.371	1.125	1.155
417	0.373	0.361	1.035	1.062
418	0.364	0.352	0.957	0.980
419	0.357	0.344	0.881	0.903
420	0.351	0.339	0.823	0.844
421	0.348	0.335	0.765	0.783
422	0.341	0.330	0.701	0.718
423	0.340	0.327	0.653	0.668
424	0.338	0.325	0.605	0.618
425	0.334	0.323	0.561	0.573
426	0.334	0.323	0.515	0.526
427	0.332	0.322	0.482	0.492
428	0.332	0.320	0.442	0.451
429	0.331	0.320	0.409	0.416

Absorbance (au)				
Wavelength (nm)	PQQ	PQQ + Ca ²⁺	PQQ + UO ₂ ²⁺	PQQ + Ca ²⁺ + UO ₂ ²⁺
430	0.331	0.320	0.377	0.383
431	0.331	0.320	0.354	0.359
432	0.332	0.322	0.327	0.332
433	0.333	0.322	0.305	0.310
434	0.333	0.323	0.288	0.291
435	0.335	0.324	0.273	0.276
436	0.335	0.325	0.255	0.257
437	0.338	0.327	0.239	0.240
438	0.338	0.329	0.230	0.229
439	0.340	0.330	0.218	0.215
440	0.340	0.331	0.209	0.204
441	0.343	0.333	0.200	0.195
442	0.343	0.335	0.193	0.188
443	0.345	0.336	0.183	0.179
444	0.346	0.338	0.172	0.169
445	0.346	0.340	0.163	0.162
446	0.348	0.340	0.158	0.157
447	0.350	0.343	0.152	0.152
448	0.351	0.343	0.147	0.147
449	0.351	0.345	0.143	0.143
450	0.352	0.346	0.139	0.139
451	0.353	0.347	0.135	0.135
452	0.353	0.349	0.131	0.131
453	0.355	0.350	0.128	0.128
454	0.355	0.351	0.126	0.127
455	0.356	0.352	0.124	0.124
457	0.358	0.355	0.119	0.118
458	0.358	0.356	0.117	0.118
459	0.358	0.357	0.115	0.115
460	0.359	0.357	0.115	0.114
461	0.359	0.358	0.114	0.114
462	0.359	0.359	0.113	0.113
463	0.359	0.359	0.111	0.111
464	0.360	0.361	0.111	0.111
465	0.360	0.362	0.110	0.109
466	0.359	0.363	0.108	0.108
467	0.359	0.364	0.108	0.108
468	0.360	0.364	0.107	0.107
469	0.359	0.364	0.106	0.106
470	0.359	0.365	0.105	0.105
471	0.358	0.365	0.105	0.104
472	0.358	0.366	0.104	0.104
473	0.358	0.367	0.105	0.104
474	0.357	0.367	0.106	0.103
475	0.357	0.367	0.108	0.102

Absorbance (au)				
Wavelength (nm)	PQQ	PQQ + Ca ²⁺	PQQ + UO ₂ ²⁺	PQQ + Ca ²⁺ + UO ₂ ²⁺
476	0.355	0.367	0.109	0.102
477	0.356	0.366	0.109	0.101
478	0.355	0.367	0.109	0.102
479	0.354	0.367	0.106	0.100
480	0.354	0.366	0.104	0.101
481	0.352	0.366	0.102	0.101
482	0.351	0.366	0.100	0.100
483	0.349	0.366	0.100	0.100
484	0.350	0.365	0.100	0.101
485	0.349	0.365	0.100	0.102
486	0.346	0.364	0.099	0.102
487	0.346	0.362	0.099	0.103
488	0.346	0.363	0.100	0.104
489	0.344	0.361	0.098	0.103
490	0.341	0.360	0.097	0.102
491	0.340	0.359	0.098	0.101
492	0.339	0.358	0.098	0.099
493	0.339	0.357	0.098	0.098
494	0.336	0.355	0.097	0.097
495	0.334	0.354	0.096	0.096
496	0.332	0.352	0.096	0.094
497	0.331	0.351	0.097	0.095
498	0.328	0.350	0.096	0.094
499	0.326	0.348	0.096	0.095
500	0.324	0.347	0.095	0.093

APPENDIX J

ADDITIONAL MASS SPECTRA AND EXPERIMENTAL CONDITIONS

In this appendix, the mass spectra discussed, but not included, in Chapter 4 are presented. Spectra were collected using an electrospray-ionization mass spectrometer (Agilent Ion Trap 6300, model G2440DA). All mixtures were added to a concentration of 10 μ M. PQQ was added as the disodium salt (Fisher Scientific, Fair Lawn, NJ). UO_2^{2+} was added as the Cl salt (International Bio-Analytical Industries Inc., Boca Raton, Florida), as was Ca^{2+} (Fisher Scientific, Fair Lawn, NJ). Samples were prepared in nano-pure water (17.5 Ω), and were injected at a flow rate of 0.3 mL/hr. Nitrogen served as the carrier gas at a flow rate of 8.0 L/min. All spectra were obtained in negative mode, and the ion spray temperature and pressure were 365°C and 75 psi, respectively.

Figure 33 shows the spectrum of the nano-pure water, which served as solvent in all subsequent treatments. Figures 34 and 35 contain the spectrum of the 10 μ M Ca^{2+} and UO_2^{2+} solutions, respectively. Figure 36 shows the spectrum of the PQQ + Ca^{2+} system. Figure 37 shows the spectrum of the PQQ + UO_2^{2+} system. Finally, Figure 38 shows the spectra of the PQQ + Ca^{2+} + UO_2^{2+} system.

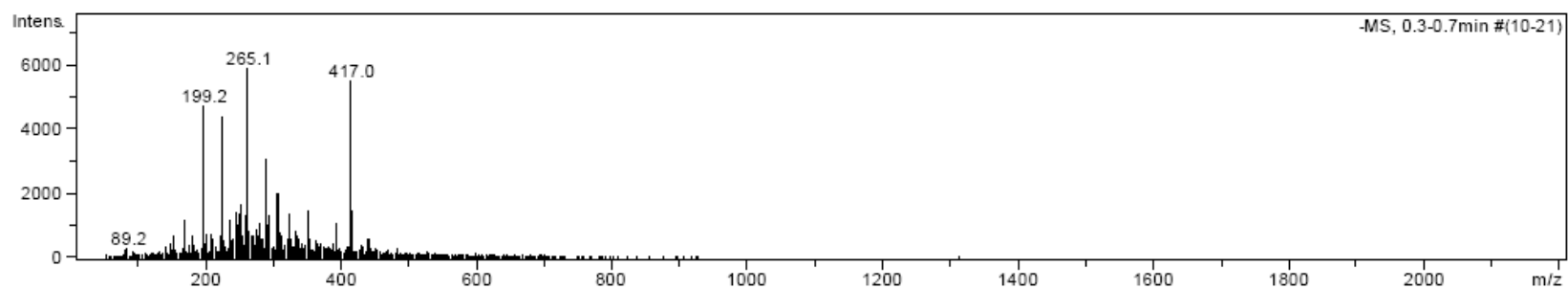


Figure 33. Spectrum of nano-pure water only.

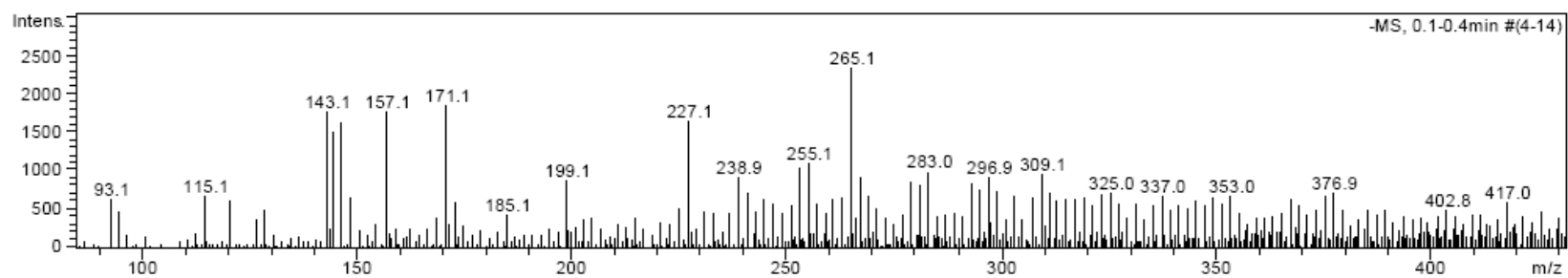


Figure 34. Spectrum of 10 μM Ca^{2+} solution.

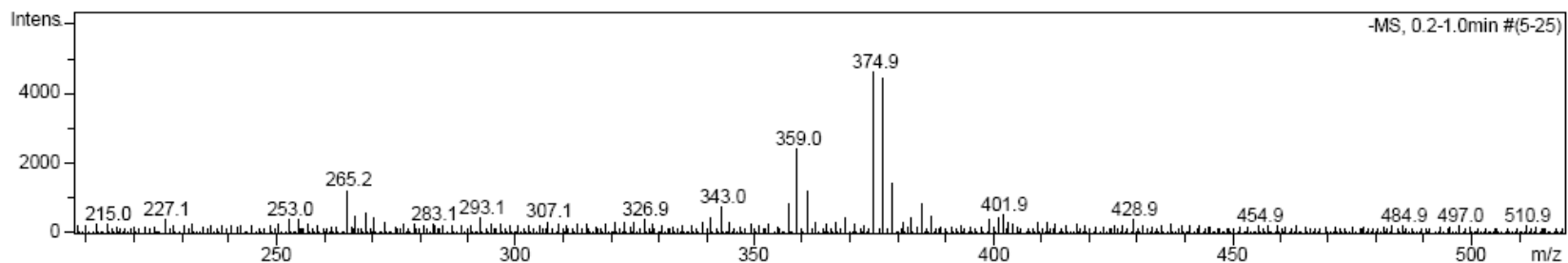


Figure 35. Spectrum of 10 μM UO_2^{2+} solution.

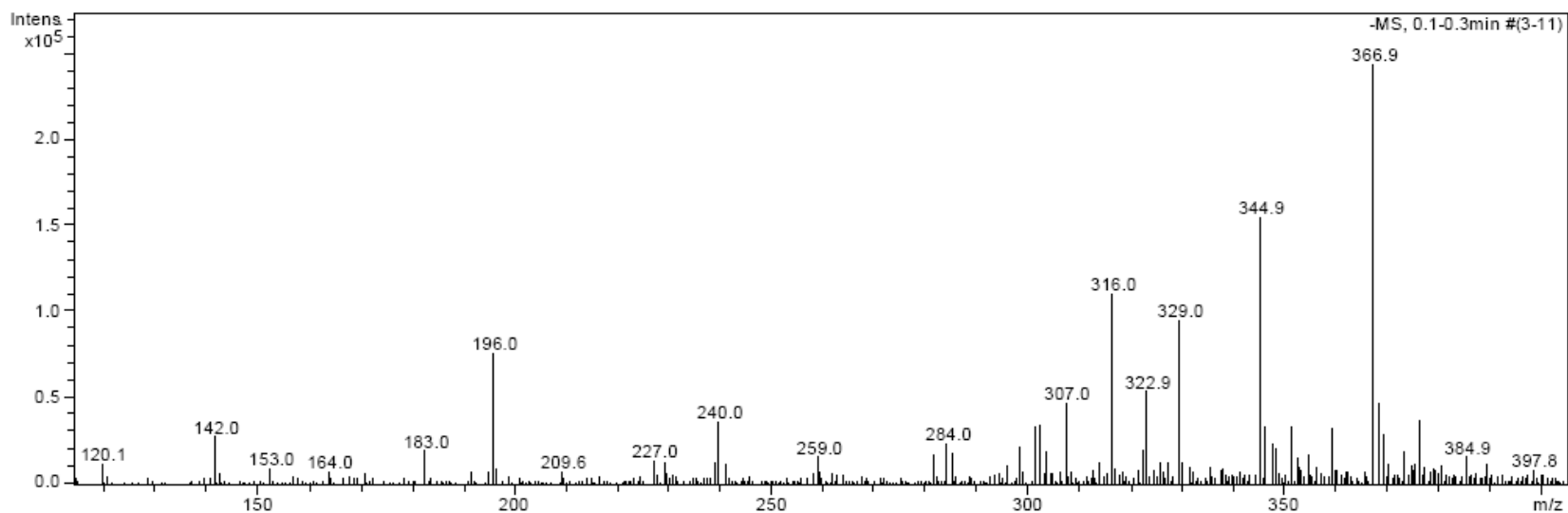


Figure 36. Spectrum of the PQQ + Ca^{2+} system.

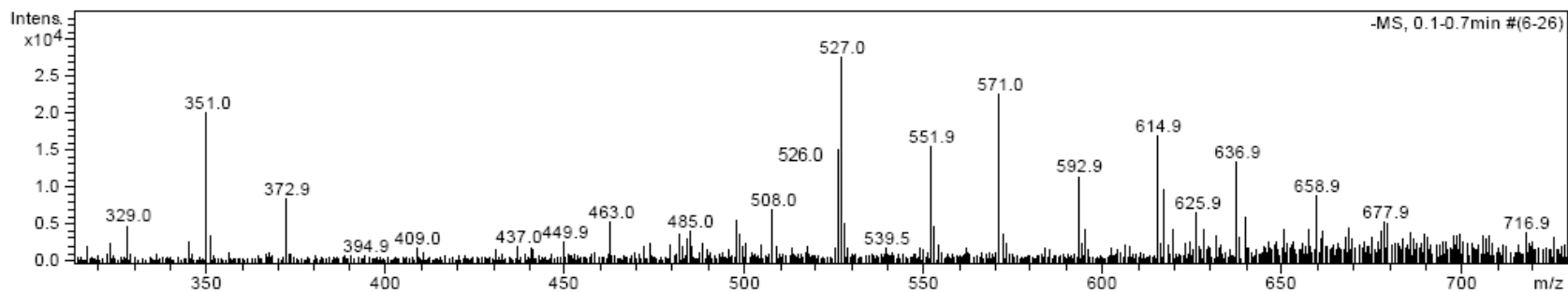


Figure 37. Spectrum of the PQQ + UO₂²⁺ system.

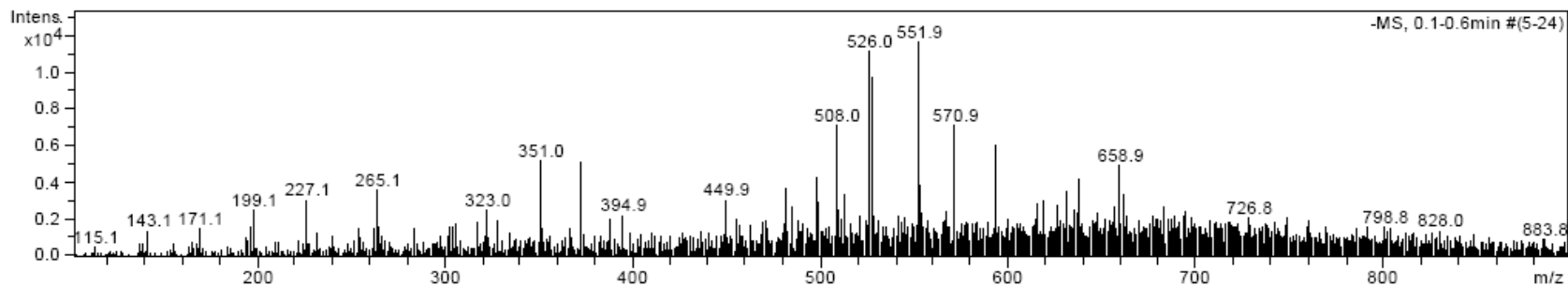


Figure 38. Spectrum of the PQQ + Ca²⁺ + UO₂²⁺ system.

APPENDIX K

EFFECT OF URANIUM ON AEROBIC ETHANOL METABOLISM OF
PSEUDOMONAS AERUGINOSA PAO1 UNDER NON-GROWTH CONDITIONS

Experiments were performed to investigate UO_2^{2+} inhibition of aerobic ethanol oxidation by *Pseudomonas aeruginosa* PAO1 under non-growth conditions. It was hypothesized that if UO_2^{2+} can acutely inhibit growth of PAO1 by inhibiting PQQ-dependent quinoprotein ethanol dehydrogenase, UO_2^{2+} toxicity could also be demonstrated under non-growth conditions as measured by a decrease in ethanol oxidation rates. In addition, it was hypothesized that UO_2^{2+} can inhibit both previously active PQQ-dependent ethanol dehydrogenase and newly expressed enzyme. Data is presented in this appendix in support of both hypotheses.

Liquid cultures of PAO1 were grown as described in Chapter 3. To test the ability of UO_2^{2+} to inhibit ethanol oxidation by previously active enzyme, cells were grown aerobically in 50 mL of simulated groundwater media (SGW) amended with 7.5 mM ethanol at 20°C in 250 mL baffled shaker flasks rotating at 100 rpm. Upon reaching mid-exponential growth phase, cells were centrifuged ($6,000 \times g$ for 20 min) and washed three times in fresh non-growth media containing 7.5 mM ethanol. Non-growth media refers to SGM lacking nitrogen and phosphorous. Cells were then suspended in 25 mL of non-growth media to a cell protein concentration of 17 ± 2 mg-protein/L (measured as described in App C) in 125 mL serum bottles sealed with butyl stoppers and crimped with aluminum seals. Bottles were shaken at 100 rpm at 20°C. When appropriate, UO_2^{2+} was added as filter sterile (0.2 μm) UO_2Cl_2 (International Bio-Analytical Industries Inc., Boca Raton, Florida) to a concentration of 20 μM . Samples were periodically removed with 1 mL syringes and filtered (0.2 μm) to measure the aqueous ethanol concentration and calculate ethanol oxidation. To test the ability of UO_2^{2+} to inhibit ethanol oxidation

by newly expressed PQQ-dependent ethanol dehydrogenase, an identical procedure was followed, except that cells were initially grown on 5 mM lactate instead of ethanol. Ethanol was measured using a Hewlett Packard 5890 series II gas chromatograph according to Wu, et al. (1991) (1). Ethanol standards were prepared in non-growth SGM. The ethanol peak retention time was between 3.0 and 3.2 minutes. Each experiment was performed in replicate, and data points correspond to the average of replicate measurements. Reported errors correspond to 95% confidence intervals. Cell free and ethanol free controls were performed in parallel, each with negative results.

Table 28 shows the peak areas (au) of each of the ethanol standards used, and Figure 39 shows the standard curve generated based on this data.

Table 29 shows peak area and corresponding ethanol concentrations in liquid cultures of PAO1 aerobically oxidizing ethanol both in the presence and absence of 20 μM UO_2^{2+} . Cells in this experiment were grown previously on ethanol, and were therefore utilizing previously functioning ethanol dehydrogenase enzyme. This data is plotted in Figure 40. Data points correspond to averages of replicate measurements. Error bars correspond to 95% confidence intervals. In the absence of UO_2^{2+} , PAO1 consumed all the 7.5 mM of ethanol initially present in the non-growth SGM within 23 hrs, corresponding to a rate of ethanol disappearance of 0.021 ± 0.001 mM-EtOH/(h $\times$ mg-protein/L). The presence of 20 μM UO_2^{2+} almost completely inhibited ethanol oxidation, as 87% of the ethanol originally present was still detected after 47 hrs of incubation (compared to 92% in the cell free control). This data demonstrates the ability of UO_2^{2+} to significantly inhibit the activity of intact, previously functioning enzyme.

Table 30 shows peak area and corresponding ethanol concentrations in liquid cultures of PAO1 aerobically oxidizing ethanol both in the presence and absence of 20 μM UO_2^{2+} . Cells in this experiment were grown previously on lactate, and were therefore required to synthesize ethanol dehydrogenase enzyme. This data is plotted in Figure 41. In the absence of UO_2^{2+} , PAO1 was able to consume 4.1 mM of ethanol initially present in the non-growth SGM after 49 hrs, corresponds to a rate of ethanol disappearance of $0.0049 \pm 0.0003 \text{ mM-EtOH}/(\text{h} \times \text{mg-protein/L})$. This relatively slow rate is probably due to the absence of added nitrogen or phosphorous, both of which would be required for these cells to produce the needed ethanol dehydrogenase enzyme. Cells previously grown on ethanol had no such requirement, and were therefore able to oxidize the ethanol more than 4 times faster than the cells previously grown on lactate. Similar to the cells previously grown on ethanol, the presence of 20 μM UO_2^{2+} was able to essentially prevent ethanol oxidation, as all of the ethanol originally present was still detected after 49 hrs of incubation. This data suggests that UO_2^{2+} is able to inhibit both newly synthesized quinoprotein ethanol dehydrogenase, and active enzyme.

Overall, these results demonstrate the ability of UO_2^{2+} to significantly inhibit activity of quinoprotein ethanol dehydrogenase under non-growth conditions, in support of the hypothesis stated above. This also provides further evidence in support of the proposed mechanism UO_2^{2+} described in Chapter 3.

Table 28. Peak areas (au) of each of the ethanol standards used. Data is presented graphically in Figure 1.

ppm EtOH	peak area
0	8825
50	2.51E+05
200	9.24E+05
350	1.50E+06

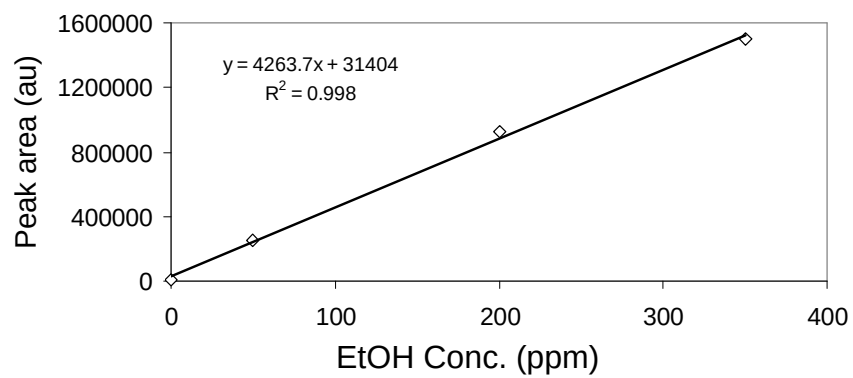


Figure 39. Standard curve and linear regression used to calculate ethanol concentrations from peak area measurements.

Table 29. Peak areas and corresponding ethanol concentrations (mM) in liquid cultures of PAO1 aerobically oxidizing ethanol in the presence and absence of $20\ \mu\text{M}\ \text{UO}_2^{2+}$. Cells were previously grown on ethanol, and therefore are utilizing functioning ethanol dehydrogenase enzyme. Data is reproduced graphically in Figure 1. Average values are averages of replicate measurements. Reported errors correspond to 95% confidence intervals.

Cells grown previously on
ethanol
U free

hours	peak area		ppm EtOH	mM EtOH	95% CI
	1	2			
0	1450000	1440000	331	7.19	0.07
3.5	1230000	1200000	281	6.10	0.21
15.5	317000	299000	72	1.57	0.13
23	1331	1254	0.30	0.01	0.00
47	2257	1077	0.52	0.01	0.01

$20\ \mu\text{M}\ \text{UO}_2^{2+}$

hours	peak area		ppm EtOH	mM EtOH	95% CI
	1	2			
0	1450000	1460000	331	7.19	0.07
3.5	1460000	1440000	333	7.24	0.14
15.5	1360000	1380000	311	6.74	0.14
23	1260000	1280000	288	6.25	0.14
47	1270000	1300000	290	6.30	0.21

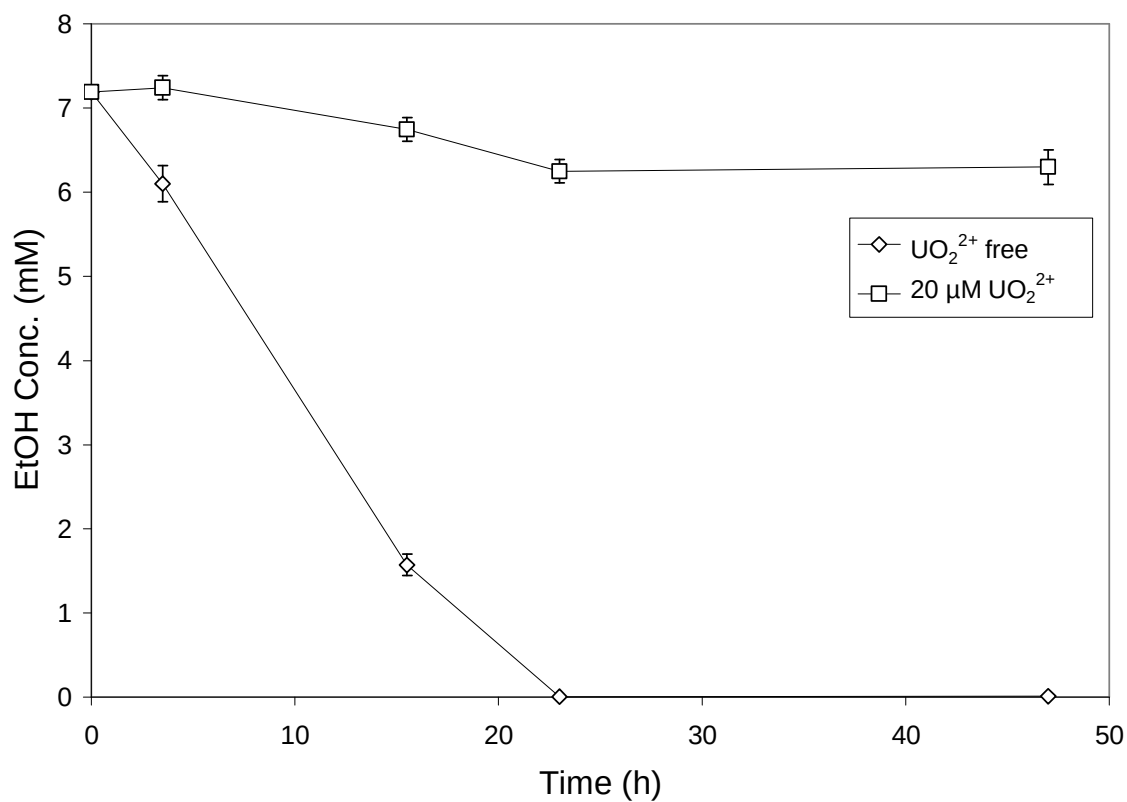


Figure 40. Ethanol oxidation by PAO1 with time in the presence and absence of 20 μM UO_2^{2+} . Cells were previously grown on ethanol, and therefore were utilizing previously functioning ethanol dehydrogenase enzyme. In the absence of UO_2^{2+} , PAO1 was able to oxidize all the 7.5 mM of ethanol initially present in 23 hrs. 20 μM of UO_2^{2+} was able to almost completely inhibit ethanol oxidation, even after 47 hrs of incubation. Data points correspond to averages of replicate measurements. Error bars correspond to 95% confidence intervals.

Table 30. Peak areas and corresponding ethanol concentrations (mM) in liquid cultures of PAO1 aerobically oxidizing ethanol in the presence and absence of $20\ \mu\text{M}\ \text{UO}_2^{2+}$. Cells were previously grown on lactate, and therefore were required to synthesize the ethanol dehydrogenase enzyme. Data is reproduced graphically in Figure 3. Average values are averages of replicate measurements. Reported errors correspond to 95% confidence intervals.

Cells grown previously on
lactate
U free

hours	peak area		ppm EtOH	mM EtOH	95% CI
	1	2			
0	1540000	1510000	352	7.64	0.21
5.5	1430000	1410000	327	7.09	0.14
17.5	1330000	1310000	304	6.60	0.14
25	1150000	1100000	263	5.70	0.36
49	712000	689000	163	3.53	0.16

$20\ \mu\text{M}\ \text{UO}_2^{2+}$

hours	peak area		ppm EtOH	mM EtOH	95% CI
	1	2			
0	1300000	1340000	297	6.45	0.28
49	1420000	1400000	324	7.04	0.14

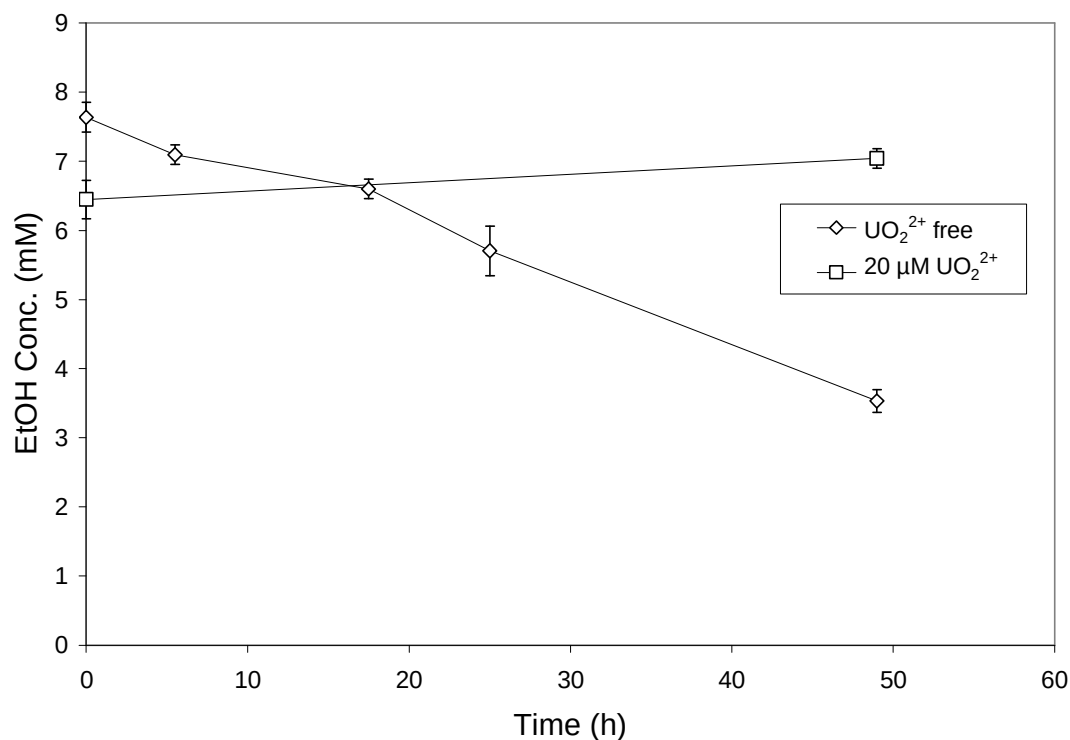


Figure 41. Ethanol oxidation by PAO1 with time in the presence and absence of $20 \mu\text{M UO}_2^{2+}$. Cells were previously grown on lactate, and therefore were required to synthesize the ethanol dehydrogenase enzyme. In the absence of UO_2^{2+} , PAO1 was able to oxidize 4.1 mM of ethanol initially present in 49 hrs. $20 \mu\text{M}$ of UO_2^{2+} was able to almost completely inhibit ethanol oxidation, even after 49 hrs of incubation. Data points correspond to averages of replicate measurements. Error bars correspond to 95% confidence intervals.

References

1. Wu W-M, Hickey R. F., and Zeikus G. J. 1991. Characterization of Metabolic Performance of Methanogenic Granules Treating Brewery Wastewater: Role of Sulfate-Reducing Bacteria. *Appl. Environ. Microbiol.* 57(12):3438-3449.

APPENDIX L

THE EFFECT OF EXCESS CALCIUM ON URANIUM TOXICITY TO
PSEUDOMONAS AERUGINOSA PAO1 DURING AEROBIC GROWTH ON ETHANOL

The sensitivity of *Pseudomonas aeruginosa* PAO1 to UO_2^{2+} toxicity during aerobic growth on ethanol in the presence of excess calcium (Ca^{2+}) was investigated. Since UO_2^{2+} exerts acute toxicity by replacing the Ca^{2+} ion present in functional quinoprotein ethanol dehydrogenase, it was hypothesized that UO_2^{2+} toxicity would be significantly mitigated by adding excess Ca^{2+} to the media, thereby allowing Ca^{2+} to outcompete UO_2^{2+} for the physiologically significant PQQ binding site. Data is presented in this appendix in support of this hypothesis.

PAO1 was grown aerobically on ethanol as described in Chapter 3. Ethanol was the only substrate used, added to a concentration of 7.5 mM, and all experiments were conducted at 20°C. The presence and absence of 20 μM UO_2^{2+} , and/or 1 mM Ca^{2+} were the only variables. Ca^{2+} was added as CaCl_2 (Fisher Scientific, Fair Lawn, NJ). UO_2^{2+} was added as UO_2Cl_2 (International Bio-Analytical Industries Inc., Boca Raton, Florida). Ethanol, Ca^{2+} , and UO_2^{2+} solutions were filter sterilized (0.2 μm) prior to use and added to previously autoclaved (30 min at 121°C) simulated groundwater media. 50 mL of prepared media was added to 250 mL baffled shaker flasks and rotated at 100 rpm. Cell concentrations were measured as the optical density (OD) of 1 mL liquid samples at 600 nm using a UV-Vis spectrophotometer (Thermo Electron Multiskan) and 48-well Corning plates. The experiment was performed in replicate, and data points correspond to the average of replicate measurements. Reported errors correspond to 95% confidence intervals.

The $\text{OD}_{600\text{nm}}$ data is presented in Table 31, and shown graphically in Figure 42. Consistent with results presented in Chapter 4, PAO1 is completely inhibited by the 20

$\mu\text{M UO}_2^{2+}$ during the first 20 hours of the experiment. However, consistent with the hypothesis stated earlier, the addition of 1 mM Ca^{2+} did significantly mitigate UO_2^{2+} toxicity. Although the lag phase was extended by about 5 hours, the cell culture density in this treatment reached 70% of the UO_2^{2+} -free treatment density after 20 hours. The presence of excess Ca^{2+} did lead to a slight increase in growth of PAO1 relative to the ethanol only system. The ethanol + UO_2^{2+} + Ca^{2+} cell density reached 65% of this enhanced density. In both cases, the addition of excess Ca^{2+} was able to allow PAO1 to recover most of its growth potential in the presence of UO_2^{2+} .

As explained in Chapter 2, UO_2^{2+} speciation largely determines its toxicity to microorganisms. It was therefore necessary to investigate the impact excess Ca^{2+} has on UO_2^{2+} speciation, since this potential might explain the observed decrease in UO_2^{2+} toxicity. Table 32 shows the species distribution of UO_2^{2+} in the media both in the presence of 1 mM Ca^{2+} and trace Ca^{2+} . Values were generated using MINTEQA2 (ver. 2.5.2). In the presence of excess Ca^{2+} , MINTEQA2 predicted the presence of Ca^{2+} - UO_2^{2+} complexes, including $\text{CaUO}_2(\text{CO}_3)_3^{2-}$ and $\text{Ca}_2\text{UO}_2(\text{CO}_3)_3$, as relatively minor components absent in the trace Ca^{2+} solution. More important is the potential impact of excess Ca^{2+} on the presence of unstable UO_2^{2+} -hydroxide complexes, which were implicated in acute UO_2^{2+} toxicity as explained in Chapter 2. MINTEQA2 predicted that excess Ca^{2+} would actually increase the relative abundance of UO_2^{2+} -hydroxide complexes by about 25%, and therefore it can be concluded that the ability of Ca^{2+} to mitigate UO_2^{2+} toxicity is not the result of corresponding changes in UO_2^{2+} speciation.

As stated in Chapter 4, the biotic ligand model (BLM) of acute metal toxicity can readily be applied to understand the impact of trace amounts of UO_2^{2+} on the growth of *Pseudomonas aeruginosa* PAO1. According to the biotic ligand model (BLM) of acute metal toxicity, acute metal toxicity results from the complexation of the metal with physiologically active binding sites, and is highly dependent on the concentration of competing ions (1, 2). In this case Ca^{2+} serves as the competing ion and, consistent with the BLM, increasing the concentration of Ca^{2+} leads to a significant decrease in UO_2^{2+} toxicity. This data is therefore consistent with the mechanism of UO_2^{2+} toxicity proposed in Chapter 4, and the BLM of acute metal toxicity in general.

Table 31. OD_{600nm} measurements of PAO1 growing aerobically on ethanol in the presence and absence of UO₂²⁺ and excess Ca²⁺. Average values represent the average of replicate treatments, and error corresponds to 95% confidence intervals of replicate treatments. Results are reproduced graphically in Figure 1.

Ethanol					Ethanol + UO ₂ ²⁺			
	OD ₆₀₀				OD ₆₀₀			
Time (h)	1	2	ave	95% CI	1	2	ave	95% CI
0	0.104	0.104	0.104	0	0.108	0.109	0.109	0.0007
3.5	0.111	0.113	0.112	0.001	0.112	0.114	0.113	0.0014
8	0.171	0.168	0.170	0.002	0.123	0.124	0.124	0.0007
20	0.429	0.418	0.424	0.008	0.119	0.125	0.122	0.0014

Ethanol + UO ₂ ²⁺ + Ca ²⁺					Ethanol + Ca ²⁺			
	OD ₆₀₀				OD ₆₀₀			
Time (h)	1	2	ave	95% CI	1	2	ave	95% CI
0	0.111	0.118	0.115	0.005	0.102	0.103	0.103	0.001
3.5	0.118	0.121	0.120	0.002	0.109	0.115	0.112	0.004
8	0.13	0.131	0.131	0.001	0.16	0.151	0.156	0.006
20	0.336	0.281	0.309	0.039	0.471	0.48	0.476	0.006

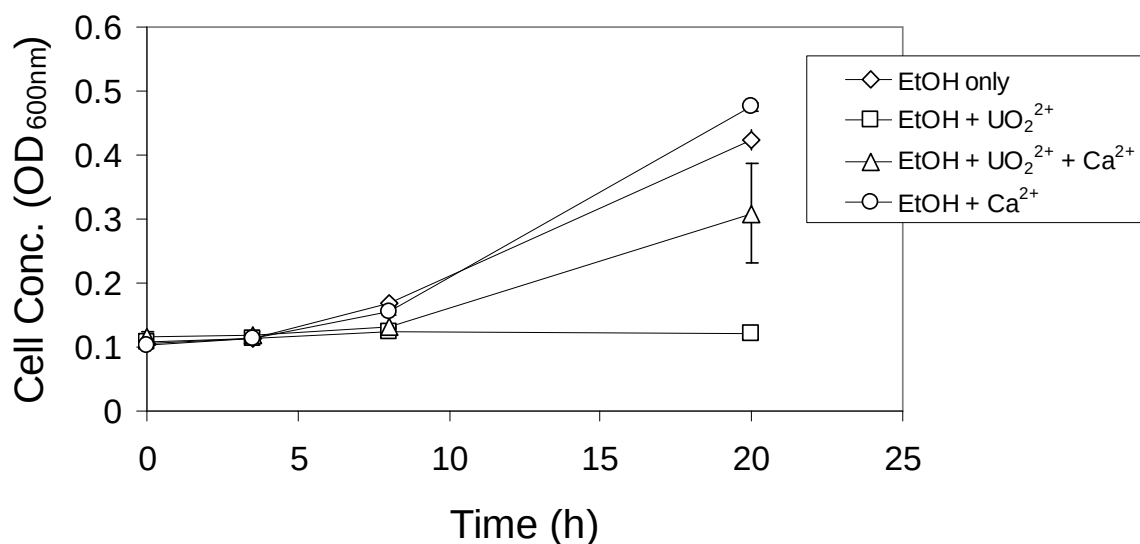


Figure 42. Graph of data presented in Table 31. The ability of excess Ca²⁺ significantly mitigated UO₂²⁺ toxicity, consistent with BLM and the mechanism of UO₂²⁺ toxicity introduced in Chapter 4. Average values represent the average of replicate treatments, and error corresponds to 95% confidence intervals of replicate treatments.

Table 32. MINTEQ (ver. 2.52) modeling results predicted UO_2^{2+} speciation distribution in the presence and absence of excess Ca^{2+} . Based on these results, the ability of Ca^{2+} to mitigate UO_2^{2+} toxicity cannot be attributed to corresponding changes in UO_2^{2+} speciation, and is therefore best understood in terms of the BLM as it relates to the mechanism of UO_2^{2+} toxicity introduced in Chapter 4.

Species	1 mM Ca^{2+}	trace Ca^{2+}
	% of total component concentration	% of total component concentration
UO_2OH^+	0.395	0.198
$(\text{UO}_2)_3(\text{OH})_5^+$	2.163	1.409
$(\text{UO}_2)_4(\text{OH})_7^+$	0.072	0.647
$\text{UO}_2(\text{OH})_3^-$	0.039	0.02
$\text{UO}_2(\text{OH})_2$ (aq)	0.449	0.234
UO_2HPO_4 (aq)	4.695	2.447
UO_2PO_4^-	22.587	10.161
$\text{Ca}_2\text{UO}_2(\text{CO}_3)_3$ (aq)	0.239	NP
$\text{CaUO}_2(\text{CO}_3)_3^{2-}$	0.175	NP
$(\text{UO}_2)_2\text{CO}_3(\text{OH})_3^-$	67.205	84.018
UO_2CO_3 (aq)	1.576	0.731
$\text{UO}_2(\text{CO}_3)_2^{2-}$	0.319	0.107
$\text{UO}_2\text{-(Lactate)}_2$ (aq)	0.018	NP
$\text{UO}_2\text{-Lactate}^+$	0.042	NP
$\text{UO}_2\text{-hydroxides}$	3.118	2.508

References

1. Di Toro, D. M. *et al.* Biotic Ligand Model of the Accute Toxicity of Metals. 1. Technical Basis. *Environ. Tox. Chem.* 20(10), 2383-2396 (2001).
2. Niyogi, S. & Wood C. M. Biotic Ligand Model, a Flexible Tool for Developing Site-Specific Water Quality Guidelines for Metals. *Environ. Sci. Technol.* 38(23), 6177-6192 (2004).

APPENDIX M

GROWTH OF *PSEUDOMONAS AERUGINOSA* PAO1 ON ETHANOL UNDER
DENITRIFYING CONDITIONS IN THE PRESENCE AND ABSENCE OF UO_2^{2+}

It was hypothesized that if low concentrations of UO_2^{2+} can significantly inhibit growth of *Pseudomonas aeruginosa* PAO1 during aerobic growth on ethanol through the binding of UO_2^{2+} to the PQQ cofactor present in the periplasmic ethanol dehydrogenase, then PAO1 should not experience significant inhibition under denitrifying conditions, as PQQ-dependent ethanol dehydrogenase is only expressed during aerobic growth (1). In this appendix, data is presented in support of this hypothesis.

PAO1 was grown under denitrifying conditions at 20°C on one of two substrates, ethanol or lactate, and in the absence or presence of 50 μM UO_2^{2+} (as UO_2Cl_2 , International Bio-Analytical Industries Inc., Boca Raton, Florida) in the simulated ground media described in Chapter 2. Substrates were added to concentrations of 15 mM-carbon. Lactate was added as the sodium salt in the form of a 60% w/w syrup and ethanol was added as the anhydrous liquid. Nitrate was added to a concentration of 10 mM as the sodium salt (NaNO_3). The lactate and NaNO_3 was supplied by Fisher (Fisher Scientific Inc., Fair Lawn, NJ), and the ethanol was supplied by Sigma-Aldrich (St. Louis, MO). Substrates, nitrate, and UO_2^{2+} solutions were filter sterilized (0.2 μm) prior to use and added to previously autoclaved (30 min at 121°C) simulated groundwater media. 50 mL of prepared media was added to 125 mL serum bottles sealed with thick butyl stoppers crimped with aluminum seals. Bottles were made anoxic by bubbling filter sterilized (0.2 μm) N_2 gas (Gases Plus, Bozeman, MT) at 15 psig through the media for 30 min. Established liquid cultures of PAO1 growing similar, UO_2^{2+} conditions served as inoculum. Cell growth was measured by reading the optical density (OD) of 1 mL liquid samples at 600 nm using a Thermo Electron Multiskan Spectrum UV-Vis

spectrophotometer and 48-well Corning plates. Nitrate utilization was inferred based on the accumulation of nitrite in the media, which was measured in filtered (0.2 μm) liquid samples by measuring the optical density at 500 nm of samples treated with a Hach Nitrivier 3 kit (Hach Co., Loveland, CO). The experiment was performed in replicate, and data points correspond to the average of replicate measurements. Reported errors correspond to 95% confidence intervals.

Table 33 shows OD₆₀₀ measurement results on both substrates, and in the presence and absence of UO_2^{2+} , and Figures 44 and 45 show this data for the lactate and ethanol systems, respectively. Similar to results obtained under aerobic growth conditions, PAO1 growth is relatively insensitive to UO_2^{2+} concentrations up to 50 μm when grown on lactate, and this trend is repeated under denitrifying conditions, as seen in Figure 44. However, unlike growth on ethanol under aerobic conditions, PAO1 growth is not inhibited significantly by 50 μm UO_2^{2+} under denitrifying conditions (Figure 45), suggesting that acute UO_2^{2+} is associated with aerobic growth, consistent with the hypothesis stated at the beginning of this appendix.

The hypothesis is further supported by the nitrite measurements, summarized in Tables 34 and 35. The direct OD₅₀₀ measurements in Table 34 were used to calculate nitrite concentrations according to the standard curve (Figure 43) generated using data in Table 36. As expected, the presence of 50 μm UO_2^{2+} did not appear to significantly impact nitrite accumulation or consumption by PAO1 regardless of substrate (Figures 44 and 45).

As stated in Chapter 4, the biotic ligand model (BLM) of acute metal toxicity can readily be applied to understand the impact of trace amounts of UO_2^{2+} on the growth of *Pseudomonas aeruginosa* PAO1. According to the biotic ligand model (BLM) of acute metal toxicity, acute metal toxicity results from the complexation of the metal with physiologically active binding sites, and therefore the toxicity of a given metal, such as UO_2^{2+} , will therefore vary depending on what specific physiological targets are available (2, 3). If, as hypothesized, PQQ is a unique physiological target of UO_2^{2+} binding, the toxicity of UO_2^{2+} should, according to BLM, vary significantly between aerobic (i.e. PQQ-dependent) and denitrifying (i.e. PQQ-independent) growth conditions in the presence of ethanol. This is unquestionably the case, and therefore the overall hypothesis presented in Chapter 3 is further supported.

Table 33. Cell concentrations, measured by optical density (OD) at 600nm, of PAO1 under denitrifying conditions with time (h) during growth on either lactate (A) or ethanol (B), both in the absence of U and in the presence of 50 μM U. Average values represent the average of replicate treatments, and error corresponds to 95% confidence intervals of replicate treatments. Results are reproduced graphically in Figures 44 and 45.

Cell conc. (OD _{600nm})								
A) Lactate	UO ₂ ²⁺ free				50 μM UO ₂ ²⁺			
Time (h)	1	2	ave	95% CI	1	2	ave	95% CI
0	0.111	0.115	0.113	0.006	0.11	0.112	0.111	0.003
17	0.138	0.144	0.141	0.008	0.132	0.13	0.131	0.003
27	0.224	0.235	0.2295	0.016	0.227	0.215	0.221	0.017
43	0.385	0.374	0.3795	0.016	0.392	0.394	0.393	0.003

B) Ethanol	UO ₂ ²⁺ free				50 μM UO ₂ ²⁺			
Time (h)	1	2	ave	95% CI	1	2	ave	95% CI
0	0.101	0.1	0.1005	0.001	0.105	0.106	0.1055	0.001
17	0.119	0.125	0.122	0.004	0.136	0.133	0.1345	0.002
27	0.175	0.152	0.1635	0.016	0.188	0.19	0.189	0.001
43	0.302	0.299	0.3005	0.002	0.32	0.341	0.3305	0.015

Table 34. Optical density at 500 nm of filtered samples (0.2 μm) treated with Nitriver 3. These values were used to calculate the nitrite concentrations shown in Table 35, according to the standard curve shown in Figure 43 and derived from the data in Table 36.

OD _{500nm}				
A) Lactate		UO ₂ ²⁺ free		50 μM UO ₂ ²⁺
Time (h)	1	2	1	2
0	0.094	0.091	0.089	0.09
17	0.131	0.141	0.132	0.132
27	0.529	0.6	0.529	0.481
43	0.092	0.092	0.091	0.091

B) Ethanol		UO ₂ ²⁺ free		50 μM UO ₂ ²⁺
Time (h)	1	2	1	2
0	0.093	0.09	0.089	0.089
17	0.145	0.141	0.135	0.147
27	0.291	0.244	0.264	0.293
43	0.168	0.26	0.223	0.123

Table 35. Nitrite accumulation and consumption by PAO1 growing on either lactate (A) or ethanol (B). Average values represent the average of replicate treatments, and error corresponds to 95% confidence intervals of replicate treatments. Results are reproduced graphically in Figures 44 and 45.

Nitrite conc. (mM)

A)									
Lactate	UO ₂ ²⁺ free				50 μM UO ₂ ²⁺				
Time (h)	1	2	ave	95% CI	1	2	ave	95% CI	
0	0.19	0.15	0.17	0.068	0.11	0.13	0.12	0.023	
17	0.79	0.95	0.87	0.227	0.81	0.81	0.81	0	
27	7.19	8.33	7.76	1.614	7.19	6.42	6.80	1.09	
43	0.16	0.16	0.16	0.000	0.15	0.15	0.15	0	

B)									
Ethanol	UO ₂ ²⁺ free				50 μM UO ₂ ²⁺				
Time (h)	1	2	ave	95% CI	1	2	ave	95% CI	
0	0.18	0.13	0.15	0.034	0.11	0.11	0.11	0	
17	1.01	0.95	0.98	0.045	0.85	1.05	0.95	0.136	
27	3.36	2.61	2.98	0.053	2.93	3.39	3.16	0.159	
43	1.38	2.86	2.12	0.119	2.27	0.66	1.46	0.114	

Table 36. Raw data used to generate an OD_{500nm} versus nitrite concentration standard curve (Figure 43). Average values represent the average of triplicate treatments, and error corresponds to 95% confidence intervals of replicate treatments.

mM nitrite	OD _{500nm}			ave	95% CI
	1	2	3		
0	0.089	0.089	0.089	0.089	0
0.5	0.115	0.115	0.119	0.116	0.005
1	0.141	0.141	0.141	0.141	0
2.5	0.221	0.22	0.226	0.222	0.006
5	0.406	0.399	0.397	0.401	0.009

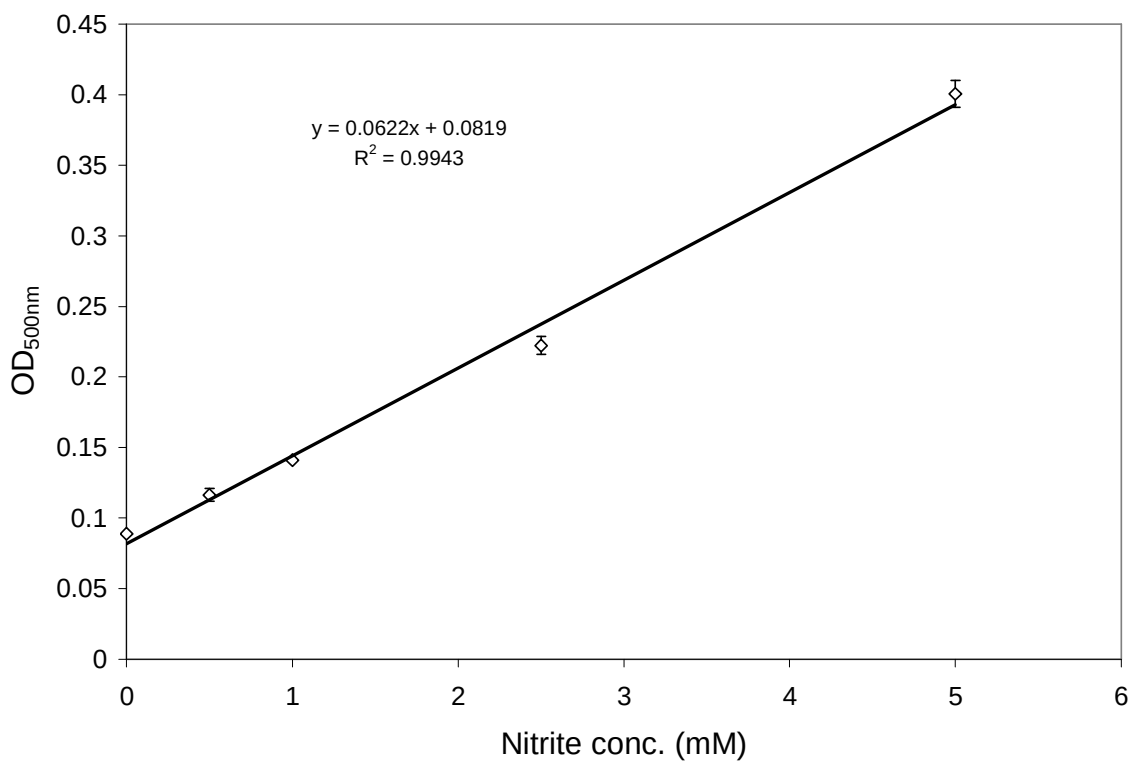


Figure 43. OD_{500nm} versus nitrite concentration standard curve derived from the data presented in Table 36. Average values represent the average of triplicate treatments, and error corresponds to 95% confidence intervals of replicate treatments.

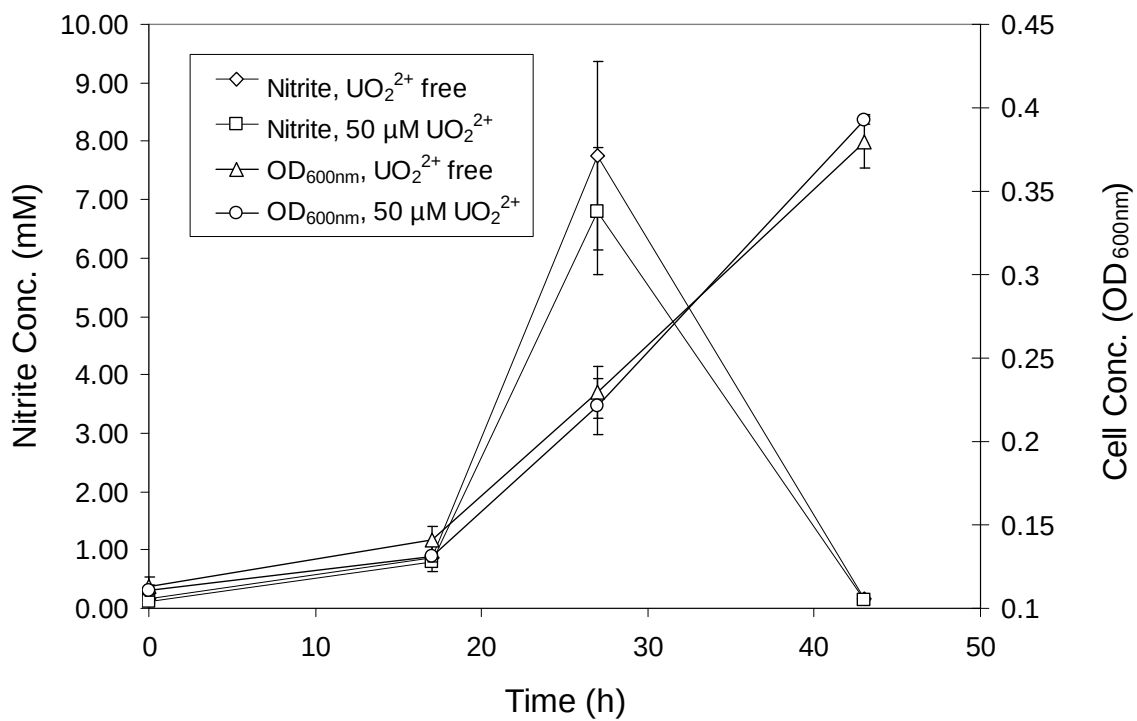


Figure 44. Growth of PAO1 on lactate under denitrifying conditions in the presence and absence of U. The presence of 50 μM UO_2^{2+} did not significantly inhibit either cell growth or nitrite accumulation or consumption, indicating minimal toxic impact.

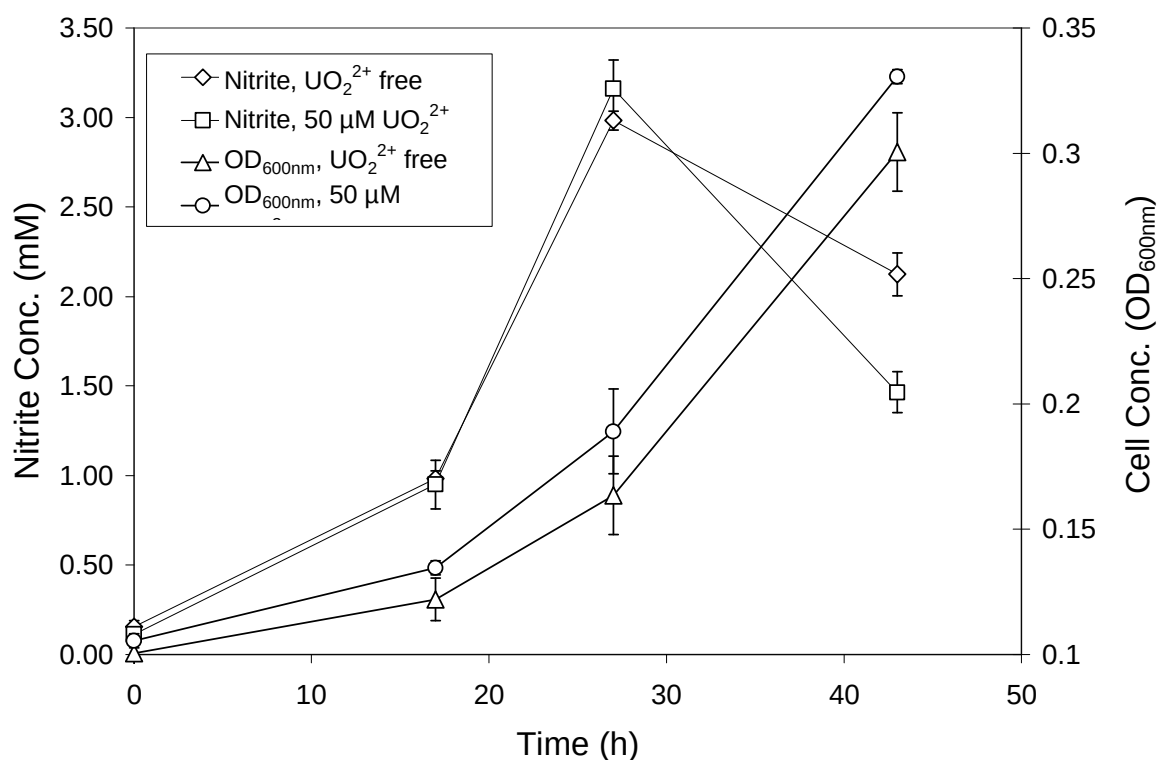


Figure 45. Growth of PAO1 on ethanol under denitrifying conditions in the presence and absence of U. Unlike growth under aerobic conditions, the presence of 50 μM UO_2^{2+} did not significantly inhibit either cell growth or nitrite accumulation or consumption, suggesting that the acute toxicity of UO_2^{2+} to PAO1 is associated with growth on ethanol only under aerobic conditions.

References

1. Görisch H. 2003. The ethanol oxidation system and its regulation in *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta*. 1647:98-102.
2. Di Toro, D. M. *et al.* Biotic Ligand Model of the Accute Toxicity of Metals. 1. Technical Basis. *Environ. Tox. Chem.* 20(10), 2383-2396 (2001).
3. Niyogi, S. & Wood C. M. Biotic Ligand Model, a Flexible Tool for Developing Site-Specific Water Quality Guidelines for Metals. *Environ. Sci. Technol.* 38(23), 6177-6192 (2004).

APPENDIX N

APPLICATION OF MOLECULAR TECHNIQUES TO ELUCIDATE THE
INFLUENCE OF CELLULOSIC WASTE ON THE BACTERIAL COMMUNITY
STRUCTURE AT A SIMULATED LOW-LEVEL WASTE SITE (PREPARED BY
ERIN K. FIELD, SETH D'IMPERIO, AMBER R. MILLER, MICHAEL R. VAN
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M. PEYTON FOR SUBMISSION TO *APPLIED AND ENVIRONMENTAL
MICROBIOLOGY*)

Introduction

The processing of nuclear materials, operation of nuclear reactors, and research and development activities at government sites, hospitals, universities, and radiochemical and radiopharmaceutical manufacturers have led to the generation of a substantial amount of low-level mixed radioactive and heavy metal wastes that have been disposed in pits, trenches, and other waste sites (2). Co-disposed with metals and radionuclides were large quantities of cellulose containing materials such as wood, paper towels, cardboard, cheesecloth, and other materials (52). These wastes result from glove box operations, decontamination, housekeeping, maintenance, and construction activities, and can constitute up to 90% of the volume of typical low-level waste (LLW) (59). While there are over 20,000 commercial users of radioactive materials (2), the Department of Energy (DOE) complex houses the majority of disposed LLW waste at sites including Savannah River, Hanford, Idaho National Laboratory (INL) and Nevada test sites (3). Prior to 2000, the DOE disposed of approximately 2 million cubic meters of LLW and has projected the disposal of an additional 10.1 million cubic meters by 2070 (3). Within the Subsurface Disposal Area at the INL alone, approximately 330 metric tons of U-238 have been buried with cellulose containing material (26, 31). While these LLW materials are generally classified as such due to their low radioactivity and metal concentrations, their large quantity suggests there is potential environmental concern if mobilization of these contaminants was to occur.

The mobility of heavy metals and radionuclides in the subsurface may be greatly affected by the decomposition of this cellulosic waste by cellulolytic or fermentative

microorganisms. A number of soil microorganisms can degrade one or more lignocellulosic components (i.e. cellulose and hemicellulose) to their respective subunits, which include cellobiose, and C-5 and C-6 sugars (i.e. xylose, mannose, and glucose) (7, 38, 43). The breakdown of cellulose itself may release the associated metals and radionuclides, potentially increasing their mobility. Additionally, fermentative bacteria can then use these cellulose breakdown products as carbon and energy sources producing a variety of fermentation products including short chain organic acids, alcohols and hydrogen (20). These fermentation products may significantly influence contaminant mobility, since organic acids can chelate metals and radionuclides potentially increasing their mobility (8, 21, 27, 44, 46). On the other hand, the work of numerous investigators has shown that these same compounds can serve as the carbon and energy source for metal and sulfate reducing bacteria that reduce and precipitate the metals and radionuclides found at these sites (1, 7, 19, 30, 39, 40, 45, 47, 51, 55, 58).

To better understand interactions between the bacterial community, cellulosic waste, and contaminants at LLW sites, the bacterial community must first be identified and the influence of the cellulosic waste on the bacterial community must be determined. Soil cores from a surrogate waste pit at the INL were collected and samples from four depths within the pit were analyzed using 16S rDNA clone libraries and high-density 16S rRNA gene microarrays (PhyloChip). The overall goal of this study was to determine how the presence of buried cellulosic waste influences the bacterial community structure found at a LLW site.

Materials and Methods

Site Description

The Cold Test Pit South (CTPS) is located at the DOE INL Radioactive Waste Management Complex (RWMC) about 50 miles west of Idaho Falls, Idaho. The CTPS was constructed in 1988 and filled with simulated wastes that conform to the historical disposal practices at the RWMC between 1953 and 1970 (57). The pit was constructed to provide a clean environment to test the implementation of innovative waste characterization, retrieval technology, and performance and operational testing of remedial action scenarios. Cardboard was used as simulated waste containers to promote rapid deterioration and simulate up to 35 years of burial in shallow land filled pits. The bottom of the CTPS was lined with a crushed sediment clay liner (Figure 46). The waste layer, designated as the wood waste layer, contains stacked cardboard boxes, drums of combustibles (scrap wood, cloth, paper, plastic and HEPA filters), metals (aluminum and steel), concrete, asphalt, glass, and simulated inorganic sludges (silica and carbonate based pastes). Evidence from previous activities in the CTPS suggests that most of the simulated waste forms were concentrated at the base of the pit between 2.4 and 4.9 m below grade. Rare earth tracers (oxides of neodymium, terbium, ytterbium or dysprosium) were added to simulate radioactive contamination. The simulated waste layer was then covered with an overlying fill soil layer using local unsaturated soil. Compaction over time reduced the size of the simulated waste layer to approximately 0.2 m.

CTPS Sampling

A truck mounted Powerprobe 9600™ (AMS, Inc., American Falls, ID) direct push sampling rig was used to obtain intact core samples from the CTPS. Soil cores spanning the depth of the pit were collected in sterile 3.2 cm diameter Lexan™ core tubes. Samples were placed in a cooler on ice for shipment to the INL laboratory where the samples were processed.

Lexan tubes were cut at four designated depths representing various layers of the pit (Figure 46). The four soil layers that were sampled were the overlying Fill soil layer (F), the Fill soil/Wood Waste interface (FW), the Wood Waste soil layer (WW), and Wood Waste/Clay interface (WC). Approximately 2.5 cm of soil was removed aseptically using a sterile spatula, then a sterile 50 ml conical centrifuge tube was used to subcore for samples from which DNA was extracted. For samples that were obtained at interfaces (FW and WC), the soil sample obtained spanned each of the upper and lower layers equally. Samples were stored at -20 °C prior to DNA extraction. Triplicate soil samples (0.3 g soil per sample) were collected for individual DNA extraction and molecular analysis from each of the four layers.

DNA Extraction and 16S rRNA Gene Amplification

DNA was extracted using the UltraClean Soil DNA Kit (MO BIO Laboratories, Inc., Carlsbad, CA). Since the WW layer soil was high in humic content, an additional clean-up step using a sephadex-based spin column was used according to instructions provided (*illustra* MicroSpin G-25 columns, GE Healthcare, UK) to remove compounds that would inhibit amplification.

PCR amplification of 16S rRNA genes was performed using 50 μ L reactions containing a final concentration of 1x PCR buffer, 0.01 mg/mL bovine serum albumin, 0.5 Units JumpStart REDTaq DNA polymerase, (Sigma-Aldrich, St. Louis, MO), 0.4 μ M 8F primer (5'-AGAGTTTGATCCTGGCTCAG-3'), and 0.4 μ M 1492R primer (5'-GGTTACCTTGTTACGACTT-3') (Integrated DNA Technologies, Coralville, IA). The reactions were heated to 94°C for 10 minutes, followed by 30 cycles of 94°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes (Applied Biosystems, GeneAmp PCR System 9700). The amplicons were checked for the correct size on the Agilent 2100 Bioanalyzer with the Agilent DNA 7500 Kit (Agilent Technologies, Waldbronn, Germany).

Cloning and Sequencing

Triplicate clone libraries were created for each soil layer using the three individual soil samples and DNA extracts obtained. 16S rRNA gene amplicons were ligated into the pCR2.1 vector using the TOPO TA Cloning Kit and transformed into Top10 competent *Escherichia coli* cells, using the instructions provided (Invitrogen, Carlsbad, CA). Transformants were plated onto Sigma S-gal/LB agar and individual colonies containing vectors with inserts were chosen based on black/white selection and used to inoculate 1mL 2xLB with kanamycin in deep well plates. The plates were incubated between 16 and 18 hours at 37°C. The plasmid DNA was purified as per manufacturer's protocol (Montage Plasmid MiniprepHTS Kit, Millipore). The average concentration of the plasmid DNA was between 100 – 300 ng/ μ L as determined using a NanoDrop, ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

The purified plasmid DNA from one clone library of each of the four soil layers was sent to Idaho State University Molecular Research Core Facility (ISU MRCF) for sequencing. The purified plasmid DNA from the other two clone libraries of each of the four soil layers was sequenced at INL. At both locations, Sanger cycle sequencing reactions with dye-terminators were prepared using between 100 and 200 ng template DNA, 1 μ L BigDye v3.1 (Applied Biosystems, Carlsbad, CA), and one of three primers: M13F (5'-GTAAAACGACGGCCAG-3'), 515F (5'-GTGCCAGCMGCCGCGGTAA-3'), or M13R (5'-CAGGAAACAGCTATGAC-3') in a reaction volume of 10 μ L (primer concentrations were 3.2 pmol/ μ L at ISU and 5 pmol/ μ L at INL). Reactions were denatured at 96°C for 1 minute, followed by 40 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. At the ISU MRCF, excess reagents and dye were removed using Millipore™-seq plates (Millipore, Billerica, MA) and DNA was analyzed on an Applied Biosystems 3130 Analyzer (Applied Biosystems, Carlsbad, CA). At INL, excess reagents and dye were removed using Performa DTRPlates (Edge Bio, Gaithersburg, MD) and DNA was analyzed on a 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA).

Sequence Analysis

Individual clones were sequenced using the forward, internal, and reverse primers, M13F, 515F, and M13R, respectively. Vector sequences were removed before assembly. Contiguous sequences were assembled using Phrap (16, 17) to make full-length 16S rRNA gene sequences. Clones were trimmed to remove poor quality regions using Phred (22) (Q<20), NAST-aligned (10), and checked for chimeras with

Bellerophon (25) all through the use of tools provided by Greengenes (12) (www.greengenes.lbl.gov). Non-chimeric sequences were compared to public databases in Greengenes and classified using the G2_Chip taxonomy classification system.

16S rRNA Gene Microarray Analysis

Amplification of the 16S rRNA gene from one of the DNA extractions obtained from each of the four soil layers was performed using 2 µg per amplification. Hybridization and subsequent analysis on a 16S rRNA gene-based microarray (PhyloChip) was carried out as previously described (11). Duplicate microarrays were analyzed for each soil layer sampled. A probe pair was scored as positive if (1) the fluorescence intensity of the perfect match probe was at least 1.3 times greater than the intensity of the mismatch probe and (2) the difference between the perfect match and mismatch intensities were 130 times greater than the square of the background intensity. An OTU was scored as positive if the positive fraction (pf) of a probe set was greater or equal to 0.92 ($pf \geq 0.92$). An OTU was scored as positive for a soil layer if the OTU met these criteria for both replicate microarrays of each layer. ARB (42) was used for the production of neighbor joining phylogenetic trees and MeV (48) for the production of heat maps.

Statistical Analysis

Statistical differences between duplicate PhyloChips and triplicate clone libraries for each layer were evaluated by Unifrac (41). Unweighted Principal Coordinates Analysis (PCoA) and lineage specific analysis were performed using Unifrac software for

both the clone library and PhyloChip NAST-aligned sequences. Before PCoA analysis, clone libraries were analyzed using DOTUR (53) (www.plantpath.wisc.edu/fac/joh/dotur.html) in which a 97% cutoff was used to group sequences into OTUs and eliminate phylogenetic weighting prior to analysis. Shannon's and Simpson's diversity indices for both the clone library and PhyloChip data sets were also calculated using DOTUR.

Nucleotide Accession Numbers

All nucleotide sequences from clone library analyses were deposited in GenBank under accession numbers GQ262819-GQ264537.

Results

Clone Library and Phylo Chip Analyses

A total of 448, 431, 382, and 458 clones from three separate clone libraries were obtained from the F, FW, WW, and WC layers, respectively, after sequences were trimmed, aligned and screened for chimeras. The complete clone library of the simulated LLW site contained 1719 clones. The triplicate clone library results for each layer were evaluated using Unifrac and were determined not to be significantly different ($p \geq 0.2$). Therefore, the triplicate libraries for each layer were combined and considered as one complete library for this study.

Duplicate PhyloChip analyses performed for each layer were also evaluated using Unifrac, determined not to be significantly different ($p \geq 0.2$), combined, and also

reported as one data set for each layer. A total of 717, 1356, 1567, and 1582 unique OTUs were scored as positive in the F, FW, WW and WC layers, respectively.

Bacterial Community Structure

Both the clone library and PhyloChip results indicated that the bacterial community profile changed with depth when viewed at the phylum level. Clone library analysis revealed that *Proteobacteria* were dominant in all four layers accounting for 29, 28, 35, and 56% of the F, FW, WW, and WC layer total clones, respectively (Figure 47A). Twelve phyla were detected in the F layer by clone library analysis, with the *Proteobacteria*, *Actinobacteria* and *Gemmatimonadetes* phyla comprising the majority of the total clones detected. These three phyla represented 332 of the 448 F clones or 74%. The FW layer contained clones from 10 different phyla, the least of any of the layers. The FW layer was comprised mostly of clones within the *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*. The *Actinobacteria* and *Bacteroidetes* combined represented 60% of the total FW layer clones. This was a significant increase in *Bacteroidetes* clones from the F layer as they were 34% of the total FW layer clones and only 1% of the total F layer clones. The WW layer contained clones from 13 different phyla, the most of any layer, and the WC layers contained clones from 12 different phyla. Additionally, both layers were comprised mainly of *Proteobacteria*, *Bacteroidetes*, and *Acidobacteria*. These three phyla represented 286 clones, 74% of the total WW layer clones, and 379 clones, 83% of the total clones in the WC layer.

The PhyloChip data also indicated a change in community profile with depth and showed greater numbers of unique OTUs with increasing depth (Figure 47B). Though the

number of unique OTUs changed with depth, four phyla were consistently dominant, and in similar ratios to each other, in all soil layers. The *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* accounted for approximately 77, 84, 82, and 81% of the total OTUs detected by PhyloChip analysis in the F, FW, WW, and WC layers, respectively. In each layer, the *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* comprised approximately 50%, 15%, 11%, and 6%, respectively, of the total OTUs detected by the PhyloChip in each soil layer.

A comparison at the OTU level between methods indicates that the PhyloChip detected significantly more OTUs than the clone libraries in all soil layers. Clone library analyses detected 191, 173, 217, and 252 unique OTUs in the F, FW, WW, and WC layers, respectively compared to the PhyloChip analyses which as previously mentioned detected 717, 1356, 1567, and 1582 unique OTUs in the same layers. A total of 2002 unique OTUs were detected by the entire study. Of these, only 10% were detected by both the clone library and PhyloChip. Another 10% were detected by the clone library only while the remaining 80% were detected by the PhyloChip only.

Bacterial Community Diversity

Shannon's and Simpson's indices both indicated greater diversity in all four soil layers by PhyloChip analysis than by clone library analysis (Table 37). The Simpson's indices calculated for both methods demonstrated a similar trend in which overall the F and FW layers had the least diversity, while the WW and WC layers had the greatest diversity.

Shannon's indices calculated using the clone library data indicated there was no significant difference in diversity between soil layers. Conversely, Shannon's indices calculated with the PhyloChip data suggested there were significant differences in diversity between layers. Shannon's indices based on PhyloChip data determined that the FW layer had the least diversity, followed by the F layer, while the WW and WC had the greatest diversity.

Soil Layer Stratification

PCoA was performed with both the clone library and PhyloChip community data sets and the results suggest that there were significant differences between the bacterial communities with depth (Figure 48). The clone library data (Figure 48A) and PhyloChip data (Figure 48B) were first analyzed separately and yielded similar results. Triplicate clone libraries and duplicate PhyloChips for each soil layer clustered with themselves, again confirming the similarities between the replicates. When comparing soil layers, the WW and WC layers grouped closely together, while the F and FW layers clustered independently from the other layers. Not surprisingly, when the clone library and PhyloChip data sets were combined and analyzed, the method used to identify the community appeared to influence the clustering of the data more heavily than the soil layer, since the PhyloChip data sets clustered together and independently of any of the clone library data (Figure 48C). The clone library data still demonstrated the same trend seen in Figure 48A: the F and FW layers each clustered by themselves, while the WW and WC layers clustered together.

Lineage specific analysis of the clone libraries was performed with Unifrac to determine which phyla were responsible for the differences between layers observed in the PCoA analysis. Multiple branch nodes were evaluated and it was determined that groups within the *Actinobacteria* and *Bacteroidetes* phyla were responsible for the majority of significant differences between layers (p value ≥ 0.01). Unifrac could not support lineage specific analysis with the PhyloChip data, due to the large number of sequences. Because *Actinobacteria* and *Bacteroidetes* phyla are known to contain cellulose degrading microorganisms (43) and were identified as groups accounting for much of the change in bacterial community structure with depth, they were evaluated further to identify how they changed with depth. While the *Proteobacteria* also accounted for some of the changes identified by lineage specific analysis, the majority of these *Proteobacteria* clones were identified as less significant (p value ≤ 0.05) than the *Actinobacteria* and *Bacteroidetes*.

Actinobacteria Phylum: By clone library analysis, there were 123, 113, 10 and 27 clones identified as *Actinobacteria* in the F, FW, WW, and WC layers, respectively, corresponding to 28, 26, 3, and 6% of the total clones detected in each layer. Results indicate a difference in the *Actinobacteria* community structure with depth when viewed at the family level. In particular, four families showed significant changes with depth based on clone abundance: *Acidimicrobiaceae*, *Glycomycetaceae*, *Micromonosporaceae* and *Streptomyces*. Lineage specific analysis identified *Acidimicrobiaceae* as responsible for some of the differences seen with the F layer when compared to the other three layers. The *Acidimicrobiaceae* family made up 33% of the total *Actinobacteria*

clones and 8.9% of the total clones detected in the F layer. An approximate 10-fold decrease was observed between the F and FW layers in the percentage of *Acidimicrobiaceae* clones of the total clones detected. No *Acidimicrobiaceae* clones were detected in the WW layer and only 3, accounting for less than 1% of the total clones detected, were detected in the WC layer (Figure 49A). The PhyloChip, however, detected the presence of *Acidimicrobiaceae* OTUs in all four soil layers suggesting they are present throughout. The *Glycomycetaceae*, *Micromonosporaceae* and *Streptomycetaceae* families had approximately 15-fold, 13-fold, and 40-fold increases in clone abundance between the F and FW layers, respectively (Figure 49B-D). This increase was followed by significant decreases in all three families between the FW and WW layer. The PhyloChip detected only one *Glycomycetaceae* OTU; however, this OTU had a pf value of 0.92 on only one FW PhyloChip which was not enough to meet the criteria needed to be counted as present. The PhyloChip detected the presence of *Micromonosporaceae* and *Streptomycetaceae* OTUs in all four layers and significantly fewer unique OTUs were detected in both cases in the F layer.

To gain a better understanding of the potential role of the *Actinobacteria* phylum in response to the presence of cellulose, families were evaluated based on whether or not they had at least one significant change between two soil layers. A significant change was defined as at least a 4-fold increase or decrease in clone numbers, which coincides with approximately a 1% change in total clone abundance, between any two layers. Thirteen families out of 33 detected met this criterion: *Acidimicrobiaceae*, *Microthrixineae*, *Frankiaceae*, *Glycomycetaceae*, *Kineosporaceae*, *Microbacteriaceae*,

Micromonosporaceae, *Streptomycetaceae*, *Thermomonosporaceae*, *Rubrobacteraceae*, and three unclassified families. These families were then differentiated based on their potential capabilities to degrade cellulose. Those that had been reported in the literature to be known cellulose degraders, cellobiose utilizers, or suggested to be cellulose degraders were grouped together (4, 5, 9, 15, 34-37, 43, 49, 60). Similarly, those families that have never been shown to degrade cellulose, utilize cellobiose nor suggested to be able to do so were also grouped together. These groups were then compared in terms of their abundance and relative diversity with depth.

The clone abundance of the non-cellulose degrading group was highest in the F layer, accounting for 18.3% of the total clones detected in this layer, and decreased approximately 5-fold between the F and FW layer (Figure 50A). There were only 3 clones from this group in the WW layer and 7 clones in the WC layer accounting for less than 2% of the total clones in both layers. Conversely, the number of clones of the reported and implied cellulose degrading group was highest in the FW layer increasing 6-fold in abundance between the F and FW layer. This group accounted for 17.9% of the total clones detected in the FW layer, decreasing in abundance in the deeper layers accounting for 1.6% of the total clones in the WW layer and 3.5% of the total clones in the WC layer. The greatest relative diversity, identified by clone library analysis, also correlated with the soil layer in which the greatest clone abundance was detected (Figure 50B). This was the F layer for the non-cellulose degrading group and the FW layer for the reported and implied cellulose degrading group. The PhyloChip also detected the greatest number of unique OTUs in the F layer for the non-cellulose degrading group,

and in the FW layer for the reported and implied cellulose degrading group (Figure 50C). However, the change in the number of unique OTUs detected by PhyloChip analysis and relative abundance between all four layers was not as great as indicated by the clone libraries suggesting clone libraries may be more sensitive to significant changes in populations than the PhyloChip. Interestingly, the PhyloChip detected a greater number of unique OTUs within the reported and implied cellulose-degrading group than the non-cellulose degrading group in all four layers. This may be due to an underestimate of the reported and implied cellulose-degrading group's presence and diversity by the clone libraries, or may be due to a larger number of probes for this group found on the PhyloChip therefore increasing its chance of detection.

Bacteroidetes Phylum: In the *Bacteroidetes* phylum 5, 146, 93, and 69 clones were detected in the F, FW, WW, and WC layers, respectively, contributing approximately 1, 34, 24, and 15% of the total clones detected in these layers. This significant increase in the number of *Bacteroidetes* clones between the F layer and the other three layers partially explains how this phylum contributes to the observed stratification between layers. Four families in particular showed significant changes in clone abundance with depth and were identified by lineage specific analysis as contributing to the stratification between layers: *Crenotrichaceae*, *Flexibacteriaceae*, *Sphingobacteriaceae*, and KSA Unclassified clones (Figure 51). Of the four families, only the KSA Unclassified clones were detected in the F layer based on clone library analyses, accounting for only 0.9% of the total clones. Additionally, the PhyloChip only detected one or two unique OTUs in each of the four families in this layer. A significant

increase in clone abundance was observed between the F and FW layers by the *Crenotrichaceae*, *Sphingobacteriaceae*, and KSA Unclassified families in which they comprised approximately 14%, 12% and 6% of the total clones detected in the FW layer, respectively. A decrease in clone abundance was observed within the *Crenotrichaceae* family beyond the FW layer while the number of unique OTUs detected by PhyloChip did not significantly change. Both the *Sphingobacteriaceae* and KSA Unclassified families significantly decreased in clone abundance between the FW and WW layers, while the PhyloChip detected either an increase or no significant change in the number of unique OTUs present in these families between the same two layers. No *Flexibacteriaceae* clones were detected in the F or FW layers, though the PhyloChip detected the presence of OTUs within this family in both layers. *Flexibacteriaceae* clones accounted for 5.5% and 5.0% of the WW and WC layer total clones, respectively. The PhyloChip also showed an increase in the number of OTUs detected between these two layers. Unlike the *Actinobacteria*, all of the families that showed significant differences between layers contain known cellulose degraders (23, 24, 29, 32, 33, 43), except for the KSA unclassified family of which no metabolic capabilities could be found in the literature. Regardless, the large number of reported and implied cellulose degrading *Bacteroidetes* families detected by clone abundance and PhyloChip analysis in the FW, WW, and WC layers suggests that there is potential for cellulose degradation in these layers.

Discussion

Clone Library and PhyloChip Comparison

Both the clone library and PhyloChip analyses yielded valuable information about the bacterial community structure and diversity at the CTPS. While 1719 clones is a substantial clone library data set, the results of the PhyloChip analyses demonstrate that even with a large number of clones, the results barely depict the total diversity that was found at the CTPS as almost 80% of the total OTUs observed were detected by the PhyloChip only. Still, the clone libraries detected 203 OTUs that the PhyloChip did not detect, and thus provide insight into the potential abundance and dominance of these organisms at the CTPS making it a valuable method to use as well.

Similar to previous studies in which both PhyloChips and clone libraries were used, the PhyloChip detected greater overall diversity and number of unique OTUs (6, 11, 18, 50, 56). As previously mentioned, there were OTUs and even entire families detected through clone library analysis that were not detected by the PhyloChip. This may be due to poor hybridization with the probe, a sequence having a stronger affinity to the mismatch probe, or the absence of these sequences in the database when the probes were designed. It is also important to point out that when comparing the presence or absence of a specific OTU between the four soil layers, there was a low percentage of matches between the two methods. While it was not surprising that a unique OTU was detected only by the PhyloChip in a soil layer, it was surprising to observe the number of unique OTUs detected in some layers by the clone libraries only and in other layers by

the PhyloChip only. This further supports the value of using these two methods to complement each other to gain more information about the bacterial community.

Low-Level Waste Site Microbial Communities

A total of 2002 unique OTUs were detected by both methods combined in all four soil layers and the dominant phyla observed (*Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Acidobacteria*, *Firmicutes*) were similar to those in other soil studies (14, 28, 54). Additionally, at least one of the methods used detected the class, and in most cases, the family containing multiple genera identified in previous studies (*Bacillus*, *Pseudomonas*, *Citrobacter*, *Clostridia*, *Azospira*, *Quadricoccus* and *Trichococcus*) focusing on LLW sites where culture techniques and small clone libraries were used to characterize the bacterial community (19, 20).

In addition to the molecular analyses discussed in this study, six bacterial isolates (members of the genera *Pseudomonas*, *Pedobacter*, *Streptomyces*, *Flavobacterium*, *Serratia*, and *Cellulomonas*) and were obtained from cellulose degrading enrichments inoculated with the soil from the FW, WW, and WC layers (Supplemental methods). All six isolates were detected at the family level by PhyloChip analysis in all three soil layers. Clone library analyses detected some of these families, such as *Enterobacteriaceae* containing the *Serratia* sp. isolate and *Sphingobacteriaceae* containing the *Pedobacter* sp. isolate, in layers from which they were not isolated. This suggests that either these organisms were present and we were unable to isolate them, or a different member of the family was present. Interestingly, a couple of the isolates, including *Streptomyces* sp., *Cellulomonas* sp., and *Flavobacterium* sp. isolates, were

obtained from soil layers in which their families were not detected by clone library analysis. These results further demonstrate limits of clone library analysis and its potential to miss much of the diversity present at the site.

Influence of Cellulose on the Bacterial Community

Significant changes in the community structure and dominant phyla were observed with depth at the CTPS by both clone library and PhyloChip analyses suggesting the presence of cellulosic waste significantly influences the bacterial community at this site. PCoA analysis also supports this hypothesis as it showed a stratification of the bacterial community occurring within the CTPS between the F, FW, and WW layers. The similarities observed between the WW and WC layer bacterial communities suggest that this part of the CTPS is not as stratified as in the shallower depths. This may be due to the presence of the clay lining in the bottom that allows for the retention of water at this depth decreasing stratification between the two soil layers.

The F layer had a low diversity overall, suggesting a more oligotrophic soil environment, most likely containing few carbon and energy sources likely supplied through downward transport during precipitation and snowmelt events. Additionally, the decrease in the number of phyla detected and low calculated diversity at the FW layer, suggests that the presence of cellulose may be a selective influence on the community at this depth where those bacteria with a metabolic advantage are dominant. The dominance of the *Actinobacteria* and *Bacteroidetes* in this layer, and specific families within these phyla that contain known or potential cellulose degraders, suggests that this layer is a potential enrichment environment for cellulose degrading microorganisms.

The WW layer of the CTPS contains large quantities of cellulosic materials. Therefore, it was hypothesized that this layer would most likely enrich for cellulose degraders. In this layer, both the clone library and PhyloChip results indicate the presence of families containing known cellulose degraders, suggesting cellulose degradation may be occurring at this depth. However, increased diversity was also observed in this layer suggesting that cellulose is likely broken down and utilized by either cellulose degrading organisms themselves or by other bacteria that rely on these breakdown products for growth. These products, readily utilized by a wide variety of microorganisms, would support a greater diversity of microorganisms in this layer. When compared to the WW and WC layers, the decreased diversity observed in the FW layer may be due to selective pressures on microorganisms in this layer, such as a lack of trace nutrients that may have been buried with the simulated waste, lack of retained water or retained breakdown products, which lead to the observed decrease in diversity in the FW layer. It is also important to note that while fungi were not studied here, we recognize that they may be catalyzing cellulose degradation at this site, and therefore may be influencing the activity and diversity of the bacterial community between the different soil layers.

We hypothesized that the *Firmicutes* would be dominant at this site since this phylum contains many known cellulose degraders (13, 43), are often dominant in soil environments (28), and are spore-formers, which is likely advantageous when fluxes of water and nutrients into the system are minimal. The PhyloChip detected a large number of *Firmicutes* OTUs in all four layers demonstrating a large relative diversity of this

phylum present; however, the clone libraries detected only 24 *Firmicutes* clones total in all four soil layers and overall the number decreased with depth. It is possible that members of this phylum are either not very abundant at this site, or the extraction and cloning method was not optimal for these organisms.

While all four layers were dominated by *Proteobacteria*, this was not surprising since the *Proteobacteria* is a large, well studied phylum containing many known members. Some members of the *Proteobacteria* such as *Pseudomonas spp.* can carry out aerobic cellulose degradation (43) and while they may play a role in cellulose degradation at this site as they were detected by both methods, they did change significantly with depth. Members of this phylum, as well as other phyla that did not change significantly with depth, may play important roles in other processes occurring in the soil such as metal cycling or the cycling of other nutrients. This may have significance in future studies which will focus on the interactions between the bacterial community and heavy metals and radionuclides found at this site.

Significance and Future Studies

The results of this study provide insight on how the presence of cellulosic waste influences the bacterial community. This is the most in-depth study to date of the bacterial community found at a LLW site. To the authors' knowledge, this is also the most in-depth study to date using both clone libraries and PhyloChip analyses to identify the bacterial community found in any one soil environment due to the large clone library size, numerous PhyloChips and evaluation of the site at multiple depths. Multi-depth sampling, such as that performed in this study, can identify potentially important changes

in the microbial community that may otherwise be overlooked. This will lead to the ability to better define and identify the potential roles different microorganisms have in metal mobility at these LLW sites and better design remediation processes that may be needed at these sites in the future. Specifically for our future studies, these results provide an extensive baseline of the bacterial community present and how the presence of cellulose influences its structure. This will allow us to better identify which organisms may be playing a key role in metal mobility in column studies where the both the heavy metal and radionuclide mobility as well as changes in the microbial community will be studied.

Table 37. Shannon and Simpson's Indices Calculated Using Clone Library and PhyloChip Data for Each Soil Layer

Layer	Shannon's Index		Simpson's Index	
	CL (95% CI)	PC (95% CI)	CL	PC
F	5.56 (± 0.090)	6.21 (± 0.062)	3.40E-03	6.00E-05
FW	5.61 (± 0.093)	5.86 (± 0.074)	3.10E-03	6.20E-05
WW	5.67 (± 0.071)	7.10 (± 0.040)	1.70E-03	3.70E-05
WC	5.72 (± 0.084)	7.03 (± 0.041)	2.20E-03	3.40E-05

(CL) Clone Library, (PC) PhyloChip, (CI) Confidence Interval, (F) Fill, (FW) Fill Waste interface, (WW) Wood Waste, (WC) Waste Clay interface.

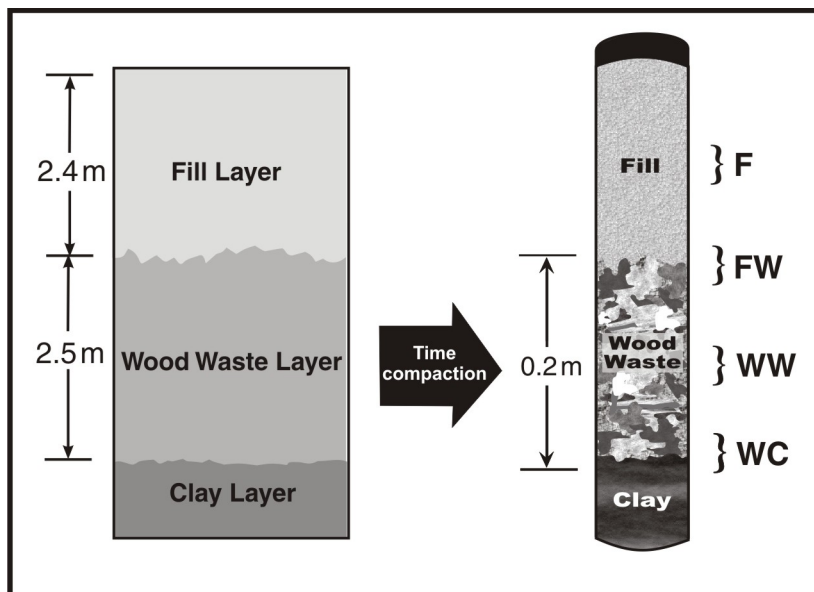


Figure 46. Schematic of the non-radioactive CTPS near the LLW site at the Idaho National Laboratory where soil samples were obtained. Brackets indicate sampling points. (F) Fill Layer, (FW) Fill Waste interface layer, (WW) Wood Waste layer, and (WC) Waste Clay interface layer.

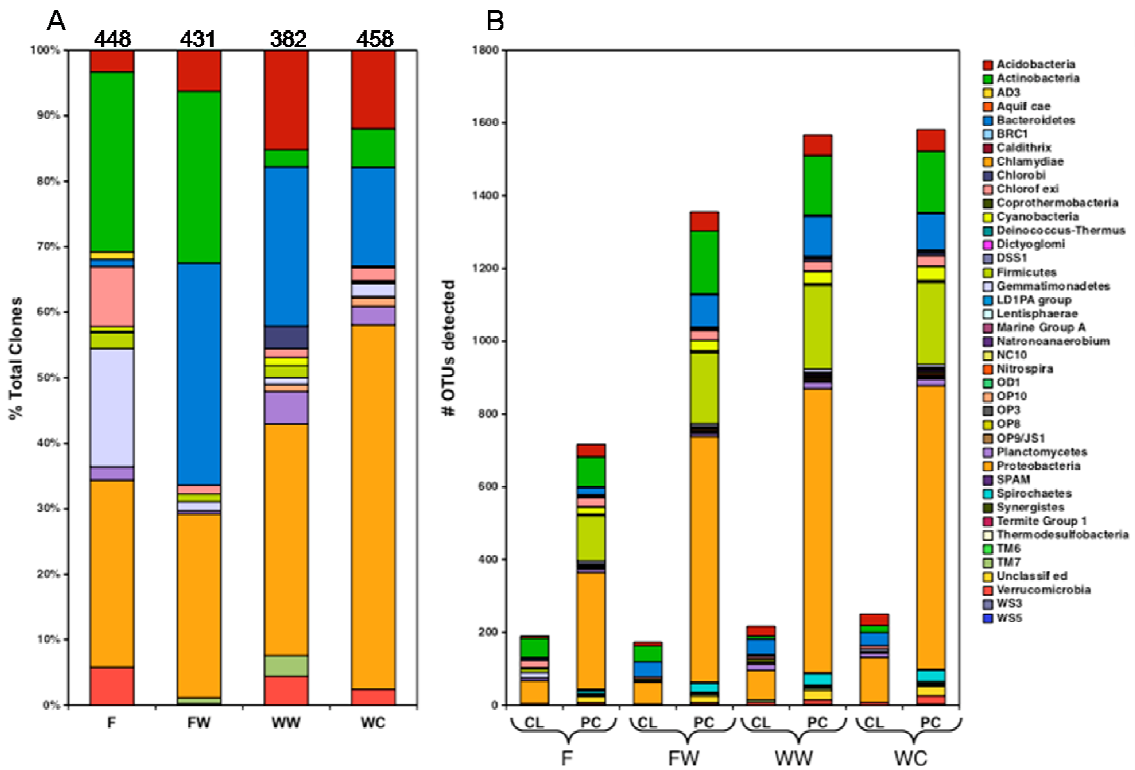


Figure 47. The bacterial community viewed at the phylum level with depth at the CTPS. (A) Percent abundance of each phylum as determined by clone library analysis with the total number of clones for that layer listed at the top of each bar. (B) Number of unique OTUs identified within each phylum based on clone library (CL) and Phylochip (PC) analyses. (F) Fill layer, (FW) Fill Waste interface layer, (WW) Wood Waste layer, (WC) Waste Clay interface layer.

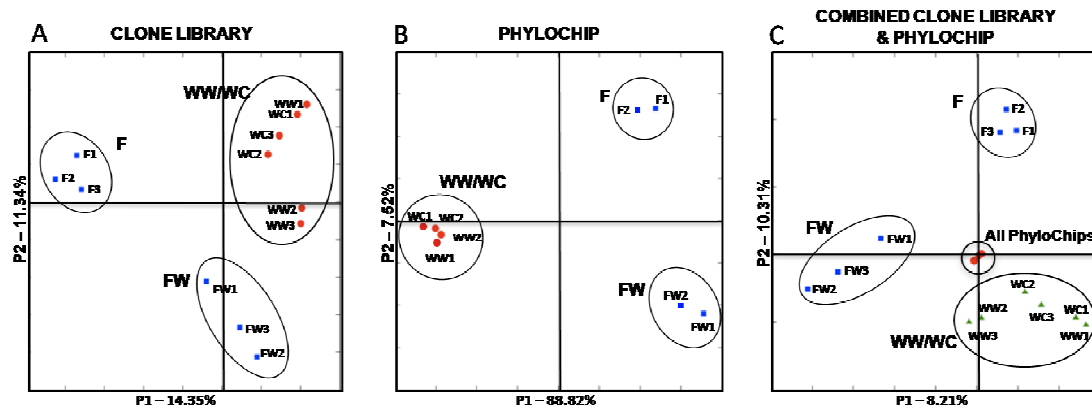


Figure 48. Principal Coordinates Analysis (PCoA) of the (A) combined clone libraries, (B) combined PhyloChip data, and (C) combined clone library and PhyloChip data. A 97% identify cutoff was used to remove replicate sequences from the clone libraries before analysis. (F) Fill layer, (FW) Fill Waste interface layer, (WW) Wood Waste layer, (WC) Waste Clay interface layer.

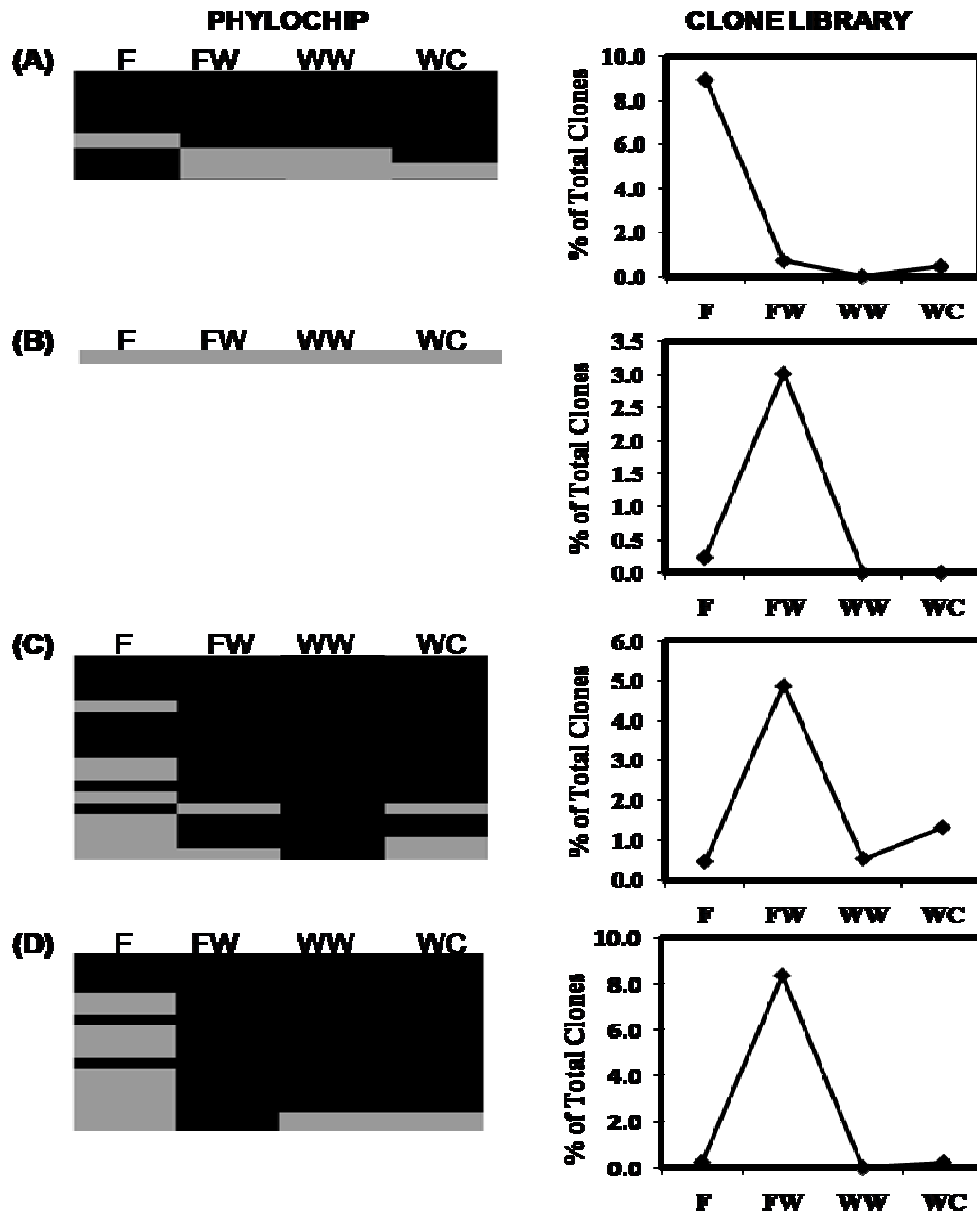


Figure 49. (A) *Acidimicrobiaceae*, (B) *Glycomycetaceae*, (C) *Micromonosporaceae*, and (D) *Streptomyces* families within the *Actinobacteria* phylum that had the most significant changes with depth as viewed by PhyloChip and clone library analyses. PhyloChip results are presented as a presence (black) absence (gray) heatmap for each OTU detected within the family. Each row represents a unique OTU. An OTU was determined present in a soil layer if the pf value was above or equal to 0.92 for both PhyloChips. Clone abundance of each family is reported as the percent of the total clones detected per soil layer. (F) Fill Layer, (FW) Fill Waste interface layer, (WW) Wood Waste layer, and (WC) Waste Clay interface layer.

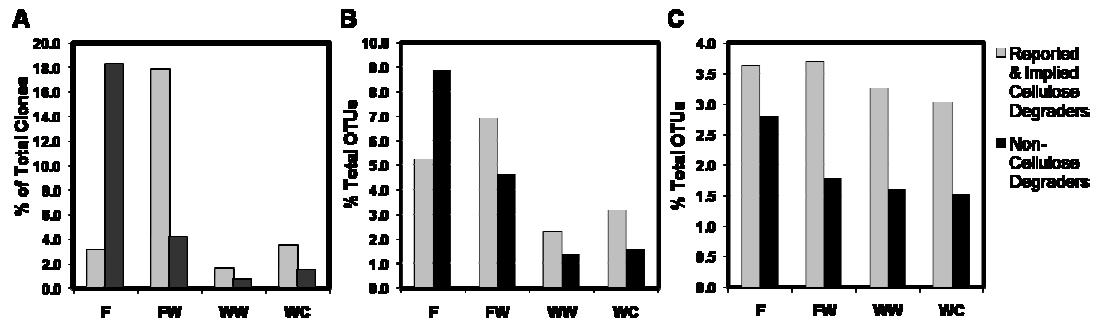


Figure 50. Focus Group Comparisons of *Actinobacteria* phylum. Families with a significant decrease in clone number between at least two layers (ex. significant change between F and FW layer) were categorized as either reported and implied cellulose degraders (families that are previously known to be cellulose degraders, cellobiose utilizers or have been suggested to be potential cellulose degraders) or non-cellulose degraders (families that have not been shown in the literature to degrade cellulose, cellobiose nor has it been suggested that they can). These two groups were then compared based on (A) clone abundance and the number of OTUs detected by (B) Clone Library and (C) PhyloChip analyses. (F) Fill Layer, (FW) Fill Waste interface layer, (WW) Wood Waste layer, and (WC) Waste Clay interface layer.

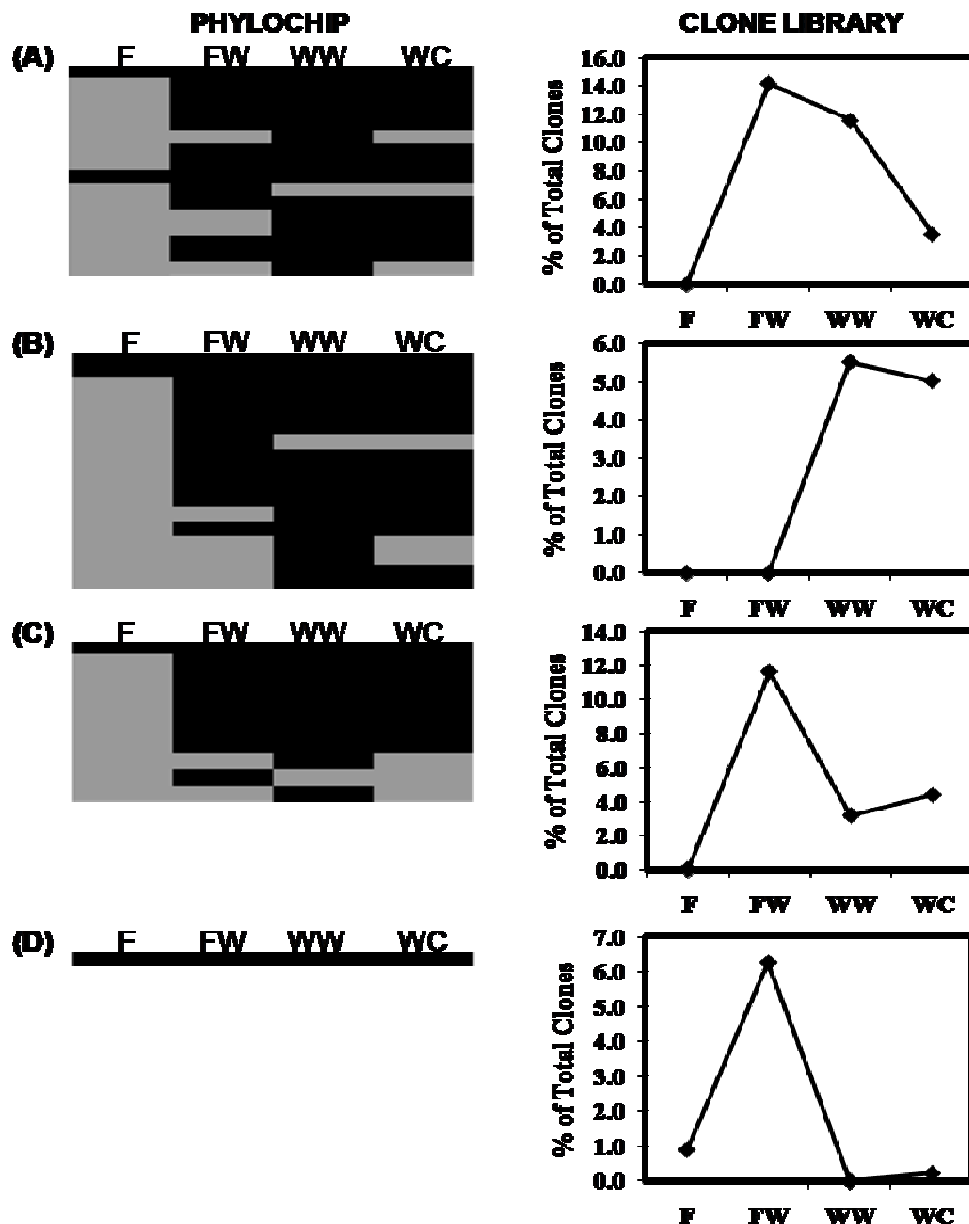


Figure 51. (A) *Crenotrichaceae*, (B) *Flexibacteraceae*, (C) *Sphingobacteriaceae*, and (D) KSA Unclassified families within the *Bacteroidetes* phylum that had the most significant changes with depth as viewed by PhyloChip and clone library analyses. PhyloChip results are presented as a presence (black) absence (gray) heatmap for each OTU detected within the family. Each row represents a unique OTU. An OTU was determined present in a soil layer if the pf value was above or equal to 0.92 for both PhyloChips. Clone abundance of each family is reported as the percent of the total clones detected per soil layer. (F) Fill Layer, (FW) Fill Waste interface layer, (WW) Wood Waste layer, and (WC) Waste Clay interface layer.

References

1. Abdelouas, A., W. Lutze, W. L. Gong, E. H. Nuttall, B. A. Strietelmeier, and B. J. Travis. 2000. Biological reduction of uranium in groundwater and subsurface soil. *Sci. Total Environ.* 250:21-35.
2. Anonymous. 1994. Radioactive waste disposal: an environmental perspective. Environmental Protection Agency.
3. Anonymous. 2000. The Current and Planned Low-Level Waste Disposal Capacity Report Revision 2. Department of Energy.
4. Bayer, E., Y. Shoham, and R. Lamed. 2006. Cellulose-decomposing Bacteria and Their Enzyme Systems, p. 578-617, *The Prokaryotes*.
5. Bentley, S., C. Corton, S. Brown, A. Barron, L. Clark, J. Doggett, B. Harris, D. Ormond, M. Quail, and G. May. 2008. Genome of the Actinomycete plant pathogen *Clavibacter michiganensis subsp. sepedonicus* suggests recent niche adaptation. *J. Bacteriol.* 190:2150-2160.
6. Brodie, E., T. Desantis, D. Joyner, S. Baek, J. Larsen, G. Andersen, T. Hazen, P. Richardson, D. Herman, T. Tokunaga, J. Wan, and M. Firestone. 2006. Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. *Appl. Environ. Microbiol.* 72:6288-6298.
7. Chew, I., J. Obbard, and R. Stanforth. 2001. Microbial cellulose decomposition in soils from a rifle range contaminated with heavy metals. *Environ. Pollut.* 111:367-375.
8. Choy, C., G. Korfiatis, and X. Meng. 2006. Removal of depleted uranium from contaminated soils. *J. Hazard. Mater.* 136:53-60.
9. Coughlan, M., and F. Mayer. 1992. The cellulose-decomposing bacteria and their enzyme systems, p. 460-516, *The Prokaryotes*.
10. DeSantis Jr, T., P. Hugenholtz, K. Keller, E. Brodie, N. Larsen, Y. Piceno, R. Phan, and G. Andersen. 2006. NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Res.* 34:394-399.

11. DeSantis, T., E. Brodie, J. Moberg, I. Zubietta, Y. Piceno, and G. Andersen. 2007. High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. *Microb. Ecol.* 53:371-383.
12. DeSantis, T., P. Hugenholtz, N. Larsen, M. Rojas, E. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. Andersen. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72:5069-5072.
13. Desvaux, M. 2005. *Clostridium cellulolyticum*: model organism of mesophilic cellulolytic clostridia. *FEMS Microbiol. Rev.* 29:741-764.
14. Elshahed, M., N. Youssef, A. Spain, C. Sheik, F. Najar, L. Sukharnikov, B. Roe, J. Davis, P. Schloss, V. Bailey, and L. Krumholz. 2008. Novelty and uniqueness patterns of rare members of the soil biosphere. *Appl. Environ. Microbiol.* 74:5422-5428.
15. Evtushenko, L., and M. Takeuchi. 2006. The Family *Microbacteriaceae*, p. 1020-1098, *The Prokaryotes*.
16. Ewing, B., and P. Green. 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities, p. 186-194, vol. 8. Cold Spring Harbor Laboratory Press.
17. Ewing, B., L. Hillier, M. Wendl, and P. Green. 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment, p. 175-185, vol. 8. Cold Spring Harbor Laboratory Press.
18. Flanagan, J., E. Brodie, L. Weng, S. Lynch, O. Garcia, R. Brown, P. Hugenholtz, T. DeSantis, G. Andersen, J. Wiener-Kronish, and J. Bristow. 2007. Loss of bacterial diversity during antibiotic treatment of intubated patients colonized with *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* 45:1954-1962.
19. Fox, J., R. Mortimer, G. Lear, J. Lloyd, I. Beadle, and K. Morris. 2006. The biogeochemical behaviour of U (VI) in the simulated near-field of a low-level radioactive waste repository. *Appl. Geochem.* 21:1539-1550.
20. Francis, A., S. Dobbs, and B. Nine. 1980. Microbial activity of trench leachates from shallow-land, low-level radioactive waste disposal sites. *Appl. Environ. Microbiol.* 40:108-113.

21. Francis, C., M. Timpson, and J. Wilson. 1999. Bench- and pilot-scale studies relating to the removal of uranium from uranium-contaminated soils using carbonate and citrate lixiviants. *J. Hazard. Mater.* 66:67-87.
22. Gordon, D., C. Abajian, and P. Green. 1998. Consed: a graphical tool for sequence finishing, p. 195-202, vol. 8. Cold Spring Harbor Laboratory Press.
23. Holmes, B. 1992. The genera *Flavobacterium*, *Sphingobacterium*, and *Weeksellia*, p. 3620-3629, *The Prokaryotes*.
24. Hreggvidsson, G., E. Kaiste, O. Holst, G. Eggertsson, A. Palsdottir, and J. Kristjansson. 1996. An extremely thermostable cellulase from the thermophilic eubacterium *Rhodothermus marinus*. *Appl. Environ. Microbiol.* 62:3047-3049.
25. Huber, T., G. Faulkner, and P. Hugenholtz. 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments, p. 2317-2319, vol. 20. Oxford Univ Press.
26. Hull, L., C. Grossman, R. Fjeld, J. Coates, and A. Elzerman. 2002. Estimating Uranium Partition Coefficients from Laboratory Adsorption Isotherms. INEEL/CON-02-00106, Idaho National Engineering and Environmental Laboratory, Idaho Falls, ID (US).
27. Humphreys, P., R. McGarry, A. Hoffmann, and P. Binks. 1997. DRINK: a biogeochemical source term model for low level radioactive waste disposal sites. *FEMS Microbiol. Rev.* 20:557-571.
28. Janssen, P. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl. Environ. Microbiol.* 72:1719-1728.
29. Johansen, J., P. Nielsen, and C. Sjöholm. 1999. Description of *Cellulophaga baltica* gen. nov., sp. nov. and *Cellulophaga fucicola* gen. nov., sp. nov. and reclassification of [*Cytophaga*] *lytica* to *Cellulophaga lytica* gen. nov., comb. nov. *Int. J. Syst. Evol. Microbiol.* 49:1231-1240.
30. Kashefi, K., and D. Lovley. 2000. Reduction of Fe (III), Mn (IV), and toxic metals at 100°C by *Pyrobaculum islandicum*. *Appl. Environ. Microbiol.* 66:1050-1056.
31. Keck, K., and R. Seitz. 2002. Potential for Subsidence at the Low-Level Radioactive Waste Disposal Area. INEEL/EXT-02-01154, Idaho National Engineering and Environmental Laboratory, Idaho Falls, ID (US).

32. Khan, S., Y. Fukunaga, Y. Nakagawa, and S. Harayama. 2007. Emended descriptions of the genus *Lewinella* and of *Lewinella cohaerens*, *Lewinella nigricans* and *Lewinella persica*, and description of *Lewinella lutea* sp. nov. and *Lewinella marina* sp. nov. *Int. J. Syst. Evol. Microbiol.* 57:2946-2951.
33. Khan, S., Y. Nakagawa, and S. Harayama. 2007. *Sediminibacter furfurosus* gen. nov., sp. nov. and *Gilvibacter sediminis* gen. nov., sp. nov., novel members of the family *Flavobacteriaceae*. *Int. J. Syst. Evol. Microbiol.* 57:265-269.
34. Kroppenstedt, R., and M. Goodfellow. 2006. The Family *Thermomonosporaceae*: *Actinocorallia*, *Actinomadura*, *Spirillospora* and *Thermomonospora*, p. 682-724, *The Prokaryotes*.
35. Kämpfer, P. 2006. The Family *Streptomycetaceae*, Part I: Taxonomy, p. 538-604, *The Prokaryotes*.
36. Labeda, D., and R. Kroppenstedt. 2004. Emended description of the genus *Glycomyces* and description of *Glycomyces algeriensis* sp nov., *Glycomyces arizonensis* sp nov. and *Glycomyces lechevalierae* sp nov. *Int. J. Syst. Evol. Microbiol.* 54:2343-2346.
37. Labeda, D., R. Testa, M. Lechevalier, and H. Lechevalier. 1985. *Glycomyces*, a new genus of the *Actinomycetales*. *Int. J. Syst. Evol. Microbiol.* 35:417-421.
38. Leschine, S. 1995. Cellulose degradation in anaerobic environments. *Annu. Rev. Microbiol.* 49:399-426.
39. Liu, C., Y. Gorby, J. Zachara, J. Fredrickson, and C. Brown. 2002. Reduction kinetics of Fe (III), Co (III), U (VI), Cr (VI), and Tc (VII) in cultures of dissimilatory metal-reducing bacteria. *Biotechnol. Bioeng.* 80:637-649.
40. Lloyd, J., C. Harding, and L. Macaskie. 1997. Tc (VII) reduction and accumulation by immobilized cells of *Escherichia coli*. *Biotechnol. Bioeng.* 55:505-510.
41. Lozupone, C., and R. Knight. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71:8228-8235.
42. Ludwig, W., O. Strunk, R. Westram, L. Richter, and H. Meier. 2004. ARB: a software environment for sequence data. *Nucleic Acids Res.* 32:1363-1371.
43. Lynd, L., P. Weimer, W. van Zyl, and I. Pretorius. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* 66:406-577.

44. Means, J., D. Crerar, and J. Duguid. 1978. Migration of Radioactive Wastes: Radionuclide Mobilization by Complexing Agents. *Science* 200:1477-1481.
45. Ortiz-Bernad, I., R. Anderson, H. Vrionis, and D. Lovley. 2004. Vanadium respiration by *Geobacter metallireducens*: novel strategy for in situ removal of vanadium from groundwater. *Appl. Environ. Microbiol.* 70:3091-3095.
46. Read, D., D. Ross, and R. Sims. 1998. The migration of uranium through Clashach Sandstone: the role of low molecular weight organics in enhancing radionuclide transport. *J. Contam. Hydrol.* 35:235-248.
47. Roh, Y., S. Liu, G. Li, H. Huang, T. Phelps, and J. Zhou. 2002. Isolation and characterization of metal-reducing *Thermoanaerobacter* strains from deep subsurface environments of the Piceance Basin, Colorado. *Appl. Environ. Microbiol.* 68:6013-6020.
48. Saeed, A., V. Sharov, J. White, J. Li, W. Liang, N. Bhagabati, J. Braisted, M. Klapa, T. Currier, and M. Thiagarajan. 2003. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 34:45-51.
49. Safo-Sampah, S., and J. Torrey. 1988. Polysaccharide-hydrolyzing enzymes of *Frankia* (*Actinomycetales*). *Plant and Soil* 112:89-97.
50. Sagaram, U., K. Deangelis, P. Trivedi, G. Andersen, S. Lu, and N. Wang. 2009. Bacterial Diversity Analysis of Huanglongbing Pathogen-Infected Citrus using PhyloChips and 16S rDNA Clone Library Sequencing. *Appl. Environ. Microbiol.* 75:1566-1574.
51. Sani, R., B. Peyton, W. Smith, W. Apel, and J. Petersen. 2002. Dissimilatory reduction of Cr (VI), Fe (III), and U (VI) by *Cellulomonas* isolates. *Appl. Microbiol. Biotechnol.* 60:192-199.
52. Saunders, J., and L. Toran. 1995. Modeling of radionuclide and heavy metal sorption around low-and high-pH waste disposal sites at Oak Ridge, Tennessee. *Appl. Geochem.* 10:673-684.
53. Schloss, P., and J. Handelsman. 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl. Environ. Microbiol.* 71:1501-1506.
54. Schloss, P., and J. Handelsman. 2006. Toward a census of bacteria in soil. *PLoS Comput. Biol.* 2:786-793.

55. Shelobolina, E., S. Sullivan, K. O'Neill, K. Nevin, and D. Lovley. 2004. Isolation, characterization, and U (VI)-reducing potential of a facultatively anaerobic, acid-resistant bacterium from low-pH, nitrate-and U (VI)-contaminated subsurface sediment and description of *Salmonella subterranea* sp. nov. Appl. Environ. Microbiol. 70:2959-2965.
56. Sunagawa, S., T. Desantis, Y. Piceno, E. Brodie, M. Desalvo, C. Voolstra, E. Weil, G. Andersen, and M. Medina. 2009. Bacterial diversity and White Plague Disease-associated community changes in the Caribbean coral *Montastraea faveolata*. ISME J. 3:512-521.
57. Thompson, E. 2002. Historical description of radioactive waste management complex (RWMC) surrogated buried waste test pits for environmental restoration (ER) waste area group 7 (WAG-7) operable unit (OU) 7-13 & 7-14. Idaho National Laboratory, Idaho Falls, ID (US).
58. Viamajala, S., B. Peyton, W. Apel, and J. Petersen. 2002. Chromate reduction in *Shewanella oneidensis* MR-1 is an inducible process associated with anaerobic growth. Biotechnol. Progr. 18:290-295.
59. Vilks, P., F. Caron, and M. Haas. 1998. Potential for the formation and migration of colloidal material from a near-surface waste disposal site. Appl. Geochem. 13:31-42.
60. Vobis, G. 2006. The Genus *Actinoplanes* and Related Genera, p. 623-653, The Prokaryotes.