Fluorescence Spectroscopy and Microscopy as Tools for Monitoring Redox Transformations of Uranium in Biological Systems.

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List of Abbreviations

CCI’s – Cation-Cation Interactions
DMRB – Dissimilatory Metal Reducing Bacteria
NADH – Nicotinamide Adenine Dinucleotide
QH$_2$ – Coenzyme H
ATP – Adenosine TriPhosphate

*et al.* - Et alii, Latin: "and others"

EPS – Extracellular polymeric substance
EXAFS – Extended X-ray Absorption Fine Structure
DFT – Density Functional Theory
XRD – X-Ray Diffraction
TEM – Transmission Electron Microscopy
XAS – X-ray Absorption Spectroscopy
XANES – X-ray Absorption Near Edge Structure
FAD – Flavin Adenine Dinucleotide
FMN – Flavin MonoNucleotide
LMCT – Ligand-to-Metal Charge Transfer
BPM – 2,2’-bipyrimidine

nIR – near InfraRed
TREM – Time Resolved Emission image Microscopy
FLIM – Fluorescence Lifetime Image Microscopy
PLIM – Phosphorescence Lifetime Image Microscopy
RNA – RiboNucleic Acid
DNA – DeoxyriboNucleic Acid
CTC – 5-cyano-2,3-ditolyl tetrazolium chloride

DAPI – 4,6-diamino-2-phenylindole

CLSM – Confocal Laser Scanning Microscopy

EPR – Electron Paramagnetic Resonance

TRLFS – Time Resolved Laser Fluorescence Microscopy

EDX – Energy Dispersive X-ray spectroscopy

MPE – MultiPhoton Excitation

2PE – 2 Photon Excitation

1PE – 1 Photon Excitation

OM – Outer Membrane

IM – Inner Membrane

GDF – Geological Disposal Facility

PBS – Phosphate Buffered Saline

LPS - LipoPolySaccharide
Abstract

Fluorescence Spectroscopy and Microscopy as Tools for Monitoring Redox Transformations of Uranium in Biological Systems.

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Science and Engineering.

The immobilisation of uranium is an important issue within the nuclear industry due to contaminated land from accidental spillage, weapons testing or mining activities. Within the environment uranium is most commonly found in the +VI oxidation state as the mobile uranyl cation \([\text{UO}_2]^{2+}\). Alternatively, the +IV oxidation state can also be found in the environment, forming either an insoluble crystalline uraninite phase, or a more soluble molecular uranium(IV) species. Many endogenous subsurface bacteria can bind and accumulate actinide ions through biosorption and can reduce mobile uranyl(VI) species down to immobile uranium(IV) compounds and mineral phases.

This work presents an investigation into the bioreduction process by two anaerobic Gram-negative bacteria, \(\text{Geobacter sulfurreducens}\) and \(\text{Shewanella oneidensis MR-1}\). Luminescence spectroscopy is used to monitor the intensity of uranyl(VI) emission \textit{in situ} over the course of a 24 hour bioreduction experiment with uranyl(VI) acetate as the electron acceptor and either acetate or lactate as the electron donor. An increase in intensity of the emission around hour three or four during the reduction, followed by an overall decrease, is attributed as the disproportionation of an unstable uranyl(V) intermediate. The role of inner and outer membrane \(c\)-type cytochromes as well as flavin secretion is also investigated using three deletion mutants of the \(S.\ oneidensis\) bacteria, which shows that in their absence, the reduction of uranyl(VI) does not occur over the course of 24 hours. The emission of uranium(IV) is also investigated during bioreduction in phosphate media and results show that emission can be observed in aqueous solutions at pH 7 pointing to the presence of a molecular product.

One photon confocal and two photon fluorescence microscopy has been utilised for the very first time to directly optically image the bioreduction of uranyl(VI) in combination with luminescence lifetime mapping. The sorption of uranyl(VI) onto the surface of the bacteria with differing lifetimes indicates a direct interaction between uranyl(VI) and surface bound \(c\)-type cytochromes, since this variation was not observed in mutant \(S.\ oneidensis\) strains where the cytochromes were not present. Combined, these results have established the applicability of optical spectroscopy and microscopy in tracking the bioreduction of uranium \textit{in situ}.

Debbie Jones
September 2016
**Declaration**

The work in this thesis was carried out at The University of Manchester between May 2013 and May 2016 under the supervision of Dr Louise Natrajan and Professor Jon Lloyd. No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university of other institute of learning.

Debbie Jones
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“The road and the tale have both been long, would you not say so? The trip has been long and the cost has been high... but no great thing was ever attained easily. A long tale, like a tall Tower, must be built a stone at a time.” Thankee-sai.

— Stephen King, The Dark Tower.
Chapter 1: Introduction

1.1 Background

The contamination of land with radionuclides such as uranium is an on-going problem for the nuclear industry. Radioactive contamination can occur via many routes; nuclear incidents, accidental leakages or spillages from current sites.\(^1\) Uranium is one of the more common radionuclides present in the environment, and causes concern due to its chemical and environmental toxicity and its long half-life of radioactivity (\(^{238}\text{U} \ t_{1/2}=4.46 \times 10^9\) years, >99%, \(^{235}\text{U} \ t_{1/2}=7.03 \times 10^8\) years, 0.72%).\(^2\) Once inside the body, uranium can cause major damage to organs such as the liver and kidneys, due to its potent nephrotoxicity.\(^3\)

In the United Kingdom, most of the nuclear industry’s legacy waste is stored at the Sellafield site.\(^4\) Due to a turbulent history involving a number of leaks and spills, radioactive contamination of the underlying soil and groundwater has occurred. This contamination is closely monitored with annual groundwater reports conducted by Sellafield Ltd. Figure 1 on the next page, shows the uranium \(^{238}\text{U}\) measurements from the year 2014.\(^5\)

In Figure 1, the majority of uranium-238 contamination is found within the ‘separations area’ (red line), and there has been no radioactivity detected beyond the site boundary (blue line). However, the site slopes gently from the inland boundary in a South Westerly direction, meaning the groundwater ultimately flows into the Irish Sea.\(^6\) This could cause problems in the future if the contamination present was soluble in the groundwater and was able to migrate beyond the site boundaries.

Within the environment, uranium is most commonly found in the +VI oxidation state as the mobile uranyl cation, (\(\text{UO}_2^{2+}\)).\(^7\) Alternatively, in reducing conditions, uranium can be found in the +IV oxidation state, generally as the insoluble black mineral uraninite.\(^8\) Achieving this reduction in oxidation state from a mobile species to an immobile species would be one method for remediation of uranium contaminated land, for example, at Sellafield,\(^9\) and is currently being actively investigated as a useful bioremediation technique.
1.2 Uranium(VI) Chemistry

Uranium(VI) (\(\text{UO}_2^{2+}\)) is found as the uranyl moiety which contains two formally triple bond U-O bonds, as shown below in Figure 2.\(^{10}\) These bonds are very short in length (~1.8 Å) and are thermodynamically stable (with a bond dissociation energy, \(\Delta H_{\text{diss}}\) of 761 kJ/mol), and are relatively chemically inert, therefore most uranyl(VI) coordination chemistry occurs around the equatorial plane.\(^{11}\) However, the formal uranyl triple bonds have been shown to be kinetically labile in acidic aqueous solutions and undergo exchange with \(^{18}\text{O}\) labelled water with a half-life of 36,000 hours (4.1 years).\(^{12}\) Uranyl(VI) is a hard Lewis acid according to the Pearson classification of hard acid soft base theory, and therefore preferentially bonds with hard donor atoms such as oxygen. This is of particular relevance to the environmental behaviour of uranyl(VI), as molecules containing oxygen donors, such as water or carbonates are very commonly found in the sub surface.\(^{13}\) Complexes can be formed with inorganic materials, such as carbonates, sulphates and phosphates, all of which bind around the equatorial plane, leaving the strong U-O bonds intact.\(^{14}\) The geochemical environment will therefore have
an important influence on the nature and concentration of the uranyl(VI) inorganic complexes found in certain areas.

\[
\left[ \begin{array}{c}
\text{O} \\
\text{U} \\
\text{O}
\end{array} \right]^{2+}
\]

**Figure 2:** The uranyl ion, showing the formally triple U-O bonds.

Multidentate ligands are very common in uranyl(VI) chemistry, and waste streams that contain radionuclides may contain multidentate ligands such as acetate, oxalate, malonate or citrate, therefore potential complexation between these ligands and uranyl(VI) may occur.\(^{15}\) This may affect the behaviour of the uranyl(VI) within the environment and cause difficulties and challenges in uranium remediation attempts.

1.3 Uranium(V) Chemistry

Uranium(V) can also form the uranyl moiety (UO\(_2^+\)), although it is inherently more unstable than the +VI oxidation state due to the presence of an unpaired electron and its propensity to disproportionate to uranyl(VI) and uranium(IV) as shown by Equation 1 below.\(^{16}\) The role of uranyl(V) within the environment is poorly understood as it is such an elusive species due to its inherent instability.\(^{17}\) However, the uranyl(V) species has been shown to play a role in environmental REDOX processes, such as bioreduction.\(^{18}\) Recent attempts to synthetically stabilize this oxidation state have resulted in an explosion of interest in uranyl(V) chemistry and have also centred around preventing the formation of Lewis-acid base uranyl-uranium O→UO\(_2^+\) cation-cation interactions (CCI’s), which are believed to aid the disproportionation through inner sphere electron transfer.\(^ {19, 20}\)

\[
2\text{UO}_2^+ + 4\text{H}^+ \leftrightarrow \text{UO}_2^{2+} + \text{U}^{4+} + 2\text{H}_2\text{O} \quad (1)
\]

1.4 Uranium(IV) Chemistry

Uranium in the +IV oxidation state differs quite considerably from its uranyl counterparts as uranium(IV) does not form the uranyl oxo-species in water as it is not sufficiently electron deficient, and unlike the uranyl oxo-species, uranium(VI) is a spherical ion, with a spherical coordination environment and a higher charge density.\(^{16}\) This results in crystalline inorganic structures and hydrolysis products forming in
environmental type conditions and contributes to the relative insolubility of uranium in the +IV oxidation state.$^{21}$

In the environment, U(IV) is commonly found as the black mineral uraninite with the formula UO$_2$, where the uranium is coordinated to eight oxygens in a cubic arrangement.$^{22}$ It is insoluble in water, but uranium(IV) as uraninite can be reoxidised to uranyl(VI) in oxic conditions and therefore these crystalline species are generally only found within the deep subsurface.$^{23}$ Other types of uranium(IV) species can be found in the environment, such as uranium(IV) phosphates, also known as ‘monomeric’ U(IV), for example $\text{U}_2\text{O}$(PO$_4$)$_2$, and $\text{U}_2$(PO$_4$)(P$_3$O$_{10}$),$^{24}$ but these are much less common.$^{25}$

1.5 Remediation Techniques

Due to the accidental and purposeful contamination of land with uranium due to nuclear fission activities, remediation techniques are being explored in an attempt to decontaminate sites. There are many remediation techniques that have been explored such as excavation and disposal, in situ chemical treatment and physical containment.$^{26}$

Excavation and disposal is the most commonly used technique across the world and involves excavating the contaminated soil and disposing of it in a nuclear licensed facility. This involves segregating the soil into various wastes depending on their contamination. This can be a time consuming method and involves many different levels of waste which can introduce new difficulty aspects to the technique.$^{26}$

In situ chemical treatment, such as chemical leaching involves the addition, via a deep well, of various chemicals to the soil, which then migrate throughout the sub surface decontaminating the soil as it travels. This radioactive liquor is then collected in a second well and disposed of as waste. This method can be very damaging to the environment and care must be taken to ensure the contaminated liquor does not travel past the collection well.$^{26}$

Physical containment involves building ‘curtain walls’ around the contaminated land to prevent contaminated water escaping the boundary. This method involves complicated engineering and is only suitable if the ground is non-permeable and contains no fractures.$^{26}$
1.6 Bioreduction

Another more simple and environmentally friendly method would be to use the indigenous bacteria within the contaminated land to bio-remediate the soil. Bacteria have been shown to reduce various metal and metalloid contaminants, including uranium and the method only requires the pumping in of a suitable electron donor to stimulate microbial population growth. This technique is currently in its infancy and more research needs to be conducted into the exact processes occurring during the “bioreduction” process before it can be considered as a practical remediation technique.

Bacteria are among the most abundant organisms on the planet with the ability to live in almost all environments, including radioactive areas. Bacteria that can gain energy by the oxidation of electron donors within the environment are called chemotrophs, and the main class of bacteria that are interesting for remediation purposes are dissimilatory metal reducing bacteria (DMRB). This class of bacteria couple the oxidation of organic matter or hydrogen to the reduction of a metal species to generate energy for respiration.

The bacteria can use the energy provided from the oxidation of an organic molecule to CO₂ and the electron(s) provided from nicotinamide adenine dinucleotide in the reduced form (NADH) and coenzyme H (QH₂) to generate adenine triphosphate (ATP) via the Krebs (or citric acid) cycle. An electron acceptor is required at the end of the process and this role can be filled by many different molecules. The electron acceptor which bacteria can gain the most energy from is oxygen. However, in the deep sub surface oxygen is not always readily available and therefore bacteria have adapted to gain energy from other sources, as can be seen in equations 2-7.

$$
C₆H₁₂O₆ + 6O₂ \rightarrow 6CO₂ + 6H₂O
\Delta G⁰ = -2872 \text{kJ mol}^{-1} \text{ glucose or } -240 \text{kJ } 2e^-
$$

(2)

$$
2.5C₆H₁₂O₆ + 12NO₃⁻ \rightarrow 6N₂ + 12CO₂ + 12OH⁻ + 9H₂O
\Delta G⁰ = -2715 \text{kJ mol}^{-1} \text{ glucose or } -226 \text{kJ } 2e^-
$$

(3)

$$
CH₃COO⁻ + 4MnO₂ + 3H₂O \rightarrow 4Mn^{2+} + 2HCO₃⁻ + 7OH⁻
\Delta G⁰ = -558 \text{kJ mol}^{-1} \text{ acetate or } -139 \text{kJ } 2e^-
$$

(4)
The first strain of bacteria shown to utilize organic compounds as an electron donor and metals as an electron acceptor, when grown in pure cultures, was *Geobacter metallireducens*. Under strict anaerobic conditions, *Geobacter* oxidised acetate to carbon dioxide and therefore reduced Fe(III) to Fe(II) according to Equation 8 below:

\[
\text{CH}_3\text{COO}^- + 8\text{Fe(OH)}_3 \rightarrow 8\text{Fe}^{2+} + 2\text{HCO}_3^- + 15\text{OH}^- + 5\text{H}_2\text{O} \\
\Delta G^\circ = -337 \text{ kJ mol}^{-1} \text{acetate or } -84 \text{ kJ } 2\text{e}^- \tag{5}
\]

\[
\text{CH}_3\text{COO}^- + 4\text{UO}_2^{2+} + 4\text{H}_2\text{O} \rightarrow 4\text{UO}_2 + 2\text{HCO}_3^- + 9\text{H}^+ \\
\Delta G^\circ = -264 \text{ kJ mol}^{-1} \text{acetate or } -66 \text{ kJ } 2\text{e}^- \tag{6}
\]

\[
\text{CH}_3\text{COO}^- + \text{SO}_4^{2-} + \text{H}_2\text{O} \rightarrow \text{H}_2\text{S} + 2\text{HCO}_3^- + \text{OH}^- \\
\Delta G^\circ = -48 \text{ kJ mol}^{-1} \text{acetate or } -12 \text{ kJ } 2\text{e}^- \tag{7}
\]

Another well-documented strain of bacteria obtained from pure cultures that shows the ability to utilize the oxidation of organic molecules coupled to the reduction of Fe(III) to gain energy for growth is *Shewanella putrefaciens* (now known as *Shewanella oneidensis MR-1*). This species oxidises lactate rather than acetate according to Equation 9 below:

\[
\text{CH}_3\text{CH(OH)COO}^- + 4\text{Fe(III)} + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{HCO}_3^- + 4\text{Fe(II)} + 5\text{H}^+ \tag{9}
\]

### 1.7 Bioreduction of Uranium

![Figure 3: Summary of the different bioreduction processes: reduction via pili on the surface of the bacteria, reduction via the electron transport chain within the bacteria and reduction via extracellular species such as flavins. Image taken from reference 18.](image)
As well as the ability to reduce Fe(III) both these bacteria have demonstrated the ability to use uranium as the terminal electron acceptor in this process. The bioreduction of uranium was first reported in 1991 by Lovley et al., who showed that certain iron reducing bacteria, such as Geobacter sulfurreducens, can obtain energy for growth by electron transport to U(VI).\textsuperscript{35} Due to it being a 2-electron reduction process, acetate oxidation coupled to uranium(VI) reduction was shown to yield almost twice the energy gained from iron(III) reduction as shown in Equation 10 below.\textsuperscript{35}

$$\text{CH}_3\text{COO}^- + 4\text{U(VI)} + 4\text{H}_2\text{O} \longrightarrow 4\text{U(IV)} + 2\text{HCO}_3^- + 9\text{H}^+ \quad (10)$$

Since then, a wide range of research has been conducted into the processes involved in the bioreduction of uranium, some of which have been summarised in Figure 3 above.\textsuperscript{18} Many different strains of bacteria have shown the ability to reduce uranyl(VI) to uranium(IV). Sulfate-reducing bacteria such as Desulfovibrio desulfuricans were shown to reduce uranium in a bicarbonate buffer with either lactate or H\textsubscript{2} as the electron donor.\textsuperscript{36} The bacteria exhibited reduction rates similar to those found with iron reducing bacteria with the formation of extracellular uranium(IV) as uraninite as the reduction product. Interestingly, sulphate was reduced concurrently with the uranium reduction indicating that in natural environment, where sulphate may be present in the groundwater, the reduction of uranium would not be impeded.\textsuperscript{36}

However, the same cannot be said for the role of nitrate contamination. Abdelous et al., found that uranium reduction by indigenous bacteria was hindered by the presence of nitrate and nitrite in the contaminated land.\textsuperscript{37} Results showed that the reduction of uranium did not begin until roughly 6 days in to the experiment and only after the reduction of nitrate to nitrite and the consequent reduction of nitrite to nitrous oxide and nitrogen gas was nearly completed.\textsuperscript{38}

The rate of uranium reduction was studied by Ulrich et al., who investigated the role of uranyl(VI) speciation on the rate of reduction. They studied four main categories of uranyl(VI) species that are to be expected in the environment; UO\textsubscript{2}(VI)-hydroxyl and UO\textsubscript{2}(VI)-organic, UO\textsubscript{2}(VI)-carbonate and CaUO\textsubscript{2}(VI)-carbonate. It was found that the rate constants for the reduction of these three categories were $k(1+2) = 10.9 \text{ h}^{-1}$, $k3 = 0.459 \text{ h}^{-1}$, and $k4 = 0.015 \text{ h}^{-1}$ respectively.\textsuperscript{39} This demonstrated that calcium present in the environment can hinder the reduction of uranyl(VI), although it is hypothesized to be due to steric effects or a higher activation barrier associated with the dissolution of the calcium-uranium-carbonate complex rather than due to a competitive reduction a
These results demonstrate the importance of the uranyl(VI) speciation within the bioreduction environment.

The role of biofilms in uranium reduction by *Geobacter sulfurreducens* has also been investigated. Biofilms occur when a group of microorganisms adhere to each other, usually within an extracellular polymeric substance (EPS) matrix. They are widely perceived to be the dominant form of microbes within the environment, and will therefore play a major role in any applications of the bioreduction process. This research suggested that biofilms reduced substantially more uranyl(VI) than individual cells, and over longer periods of time. By measuring the amount of uranium removed from solution using a fluorescent assay based technique they observed that the individual cells stopped removing uranium from the system after 12 hours, whereas the biofilm maintained a constant reduction over a 24 hour period. It was also observed that the biofilms tolerated much higher concentrations of uranium than individual cells, which could have positive repercussions in highly contaminated sites.

Although it is clear that whilst the overall bioreduction of uranyl(VI) is a two-electron process, it is not fully understood whether this occurs via a single two-electron step with reduction directly from the uranyl(VI) species to uranium(IV) or whether the process could be a one-electron reduction from uranyl(VI) to uranyl(V), which due to its instability would disproportionate to uranyl(VI) and uranium(IV), as shown in previously in equation 1.

Extended X-ray Absorption Fine Structure (EXAFS) has shown to be a useful method for determining the presence of a uranyl(V) intermediate. This was demonstrated by Renshaw *et al*., who monitored the bioreduction of uranyl(VI) acetate by *Geobacter sulfurreducens* using X-ray absorption spectroscopy. Three different oxidation states were elucidated using the uranium-oxygen bond lengths; UO$_2$(VI) and UO$_2$(V) have diagnostic axial U-O distances of 1.80 and 1.94 Å, respectively, while hydrous UO$_2$, the U(IV) material uraninit, gives U-O distances of 2.37 Å. The bond distance indicative of UO$_2$(V) can be seen after 2 hours in the bioreduction process, indicating a one-electron process forming a UO$_2$(V) intermediate.

Computational studies have also been used to model the bioreduction process within the environment, in an attempt to understand the oxidation states of uranium. Density Functional Theory (DFT) was used to investigate whether the process for *Geobacter*
`sulfurreducens` was a one- or two-electron process and concluded that the reduction involves a \( \text{UO}_2(\text{V})-\text{UO}_2(\text{V}) \) intermediate which disproportionates \textit{via} two successive protonation steps resulting in a \( \text{UO}_2(\text{VI})-\text{U(IV)} \) complex which readily dissociates forming the individual species.\(^{44}\)

Techniques such as X-Ray Diffraction (XRD) and Transmission Electron Microscopy (TEM) are often used to identify the uranium(IV) product formed.\(^{45}\) Uraninite is commonly formed and is precipitated on or near the bacteria surface. TEM studies show the localization of nanoparticulate uraninite associating with the Extracellular Polymeric Substrates (EPS) and the periplasm and outer membrane surfaces (Figure 5).\(^{46, 47}\)

![Figure 4](image)

**Figure 4.** Spectroscopic and microscopic characterization of uranium biomineralization in \textit{Myxococcus xanthus} by TEM showing nanoparticulate uraninite on the outer walls of the bacterial cell. Image taken from reference 46.

The formation of a non-uraninite U(IV) species has been observed with X-Ray Absorption Spectroscopy (XAS), suggesting that the product of uranium reduction is not always uraninite.\(^{24}\) Bioreduction studies showed that while the oxidation state of the uranium within the samples was U(IV), the X-ray Absorption Near Edge Structure (XANES) data showed a lack of a peak at 3.85 Å indicative of the U-U interaction in uraninite. Geochemical conditions and the bacteria culture media have been found to control the uranium(IV) species produced,\(^{24}\) with the presence of phosphates within the culture media promoting the formation of ‘monomeric’ uranium(IV). Some studies also suggest that ‘monomeric’ uranium(VI) is primarily associated with phosphates and carbonates on bacterial cell walls.\(^{48}\)
Further research in the *Geobacter* family suggests that extracellular pili are strongly involved in the bioreduction process. The pili can extend beyond the redox active surface of the cell forming nanowires that provide protection against the mineralisation of toxic metals within the cell envelope. In cells cultured at 25 °C, pili formation is triggered for *Geobacter sulfurreducens*, compared to 30 °C where the formation of pili is not observed. The piliated cells have been shown to remove more uranium from solution, than non-piliated cells and this is in proportion to the number of pili produced per cell. Pili are typically found on one side of the cell and extracellular precipitation of uranium(IV) has been observed on the piliated side of the cell suggesting that, when present, the pili play a role in the electron transport chain from the cell to the radionuclide.

As well as pili, cytochromes on the surface of the cell have been found to play an important role in the bioreduction process. As Figure 5 below shows, the cytochromes in *Geobacter sulfurreducens* are used in the electron transport chain to shuttle the electrons from the inner membrane of the bacteria to the outer membrane before transferring them to any pili present.

![Figure 5](image.png)

**Figure 5:** Hypothesised electron transport chain in *Geobacter sulfurreducens*. Image taken from reference 49.

Deletion of the some key outer membrane cytochromes, such as OmcE and OmcF, caused a 50-60 % decrease in the reduction of uranyl(VI) by *Geobacter sulfurreducens*. Interestingly, deletion of these cytochromes did not have an effect on the rate of iron(III) reduction, which suggests that different mechanisms for reduction are used for different metal ions.
Research into the roles of inner and outer membrane cytochromes have been extensively studied in *Shewanella oneidensis*. Borloo *et al.* have also investigated the role of OmcA and OmcB by measuring the growth curve of various mutants, OmcA<sup>−</sup>, OmcB<sup>−</sup> and with various different terminal electron acceptors.<sup>54</sup> Removal of the cytochromes severely affected the growth of bacteria using iron(III) and vanadium(V) based electron acceptors suggesting that the cytochromes play a vital role in the electron transport chain during the reduction of these metals. Interestingly, the removal of these cytochromes seemed to have no significant effect on the growth of bacteria using uranyl(VI) or selenium(VI), indicating that different reduction pathways and cytochromes may be involved.<sup>54</sup>

Other cytochromes have been identified as being involved in the electron transport chain during metal reduction in *Shewanella oneidensis*; MtrA and MtrC are periplasmic and outer membrane decahaem c-type cytochromes, the absence of which causes a decreased level of metal reduction.<sup>55</sup> Figure 6 below shows the generally accepted reduction pathway for *S. oneidensis* when reducing iron(III) oxide.<sup>55</sup>

![Figure 6](image.png)

**Figure 6:** Generally accepted reduction pathway for *S. oneidensis* and Fe(III) oxide.

Image taken from reference 52.

The secretion of flavins by *S. oneidensis* may also affect the bioreduction of uranium as extracellular electron shuttles such as flavin cofactors in flavoproteins found in the bacterial respiratory chain, can be used to enhance the reduction of metals.<sup>52</sup> Flavins are two-electron REDOX agents compared to cytochromes, which are one-electron REDOX agents, and this may have an effect on the reduction mechanisms involved.
Flavins are commonly found within biology and the biochemical source for them is the vitamin riboflavin, shown in Figure 7, along with two common flavins, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN). The use of flavins by *S. oneidensis* has also been investigated as the flavins secreted by *S. oneidensis* MR-1, can act as electron shuttles to accelerate reduction of insoluble substrates.\(^{56}\) Interestingly, this bacterium produces FAD intracellularly and is retained, while FMN and riboflavin are secreted. Kotloski *et al.*, demonstrated that roughly 75% of the bacterial respiratory capability was through electron shuttling which is mainly carried out by flavins.\(^{56}\)

![Flavins](image)

**Figure 7:** from left to right, the chemical structure of riboflavin, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN).

Due to the role of species such as cytochromes and flavins in the bacterial respiratory chain, it is generally accepted that the reduction of uranium by bacteria happens on or close to the surface of the bacteria involved.\(^{57}\) The majority of the research into uranium bioreduction has been performed with Gram-negative bacteria such as the *Geobacter* and *Shewanella* species mentioned previously. However, Gram-positive bacteria may also be important in the bioreduction of uranium.\(^{57}\)

The differences between Gram-positive and Gram-negative bacteria are shown in Table 1 below.\(^{58}\) These differences are found in the outer compartments of the bacteria, mainly with respect to the cell membrane and could have a large impact on the
interaction of uranium with the surface bound species. Gram-positive bacteria generally have a simple cell surface and can therefore provide an interesting comparison to the more complicated nature of Gram-negative bacteria.

Table 1: Comparison of the structures of Gram-positive and Gram-negative bacteria.  

<table>
<thead>
<tr>
<th>Gram-Negative Bacteria</th>
<th>Gram-Positive Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pink during gram staining</td>
<td>Purple during gram staining</td>
</tr>
<tr>
<td>Inner cell membrane</td>
<td></td>
</tr>
<tr>
<td>THIN peptidoglycan layer</td>
<td>THICK peptidoglycan layer</td>
</tr>
<tr>
<td>OM containing lipopolysaccharides and phospholipids</td>
<td></td>
</tr>
<tr>
<td>Porins on outer membrane</td>
<td></td>
</tr>
<tr>
<td>Periplasm</td>
<td>Much smaller volume periplasm</td>
</tr>
<tr>
<td>S-layer (proteins) directly attached to OM</td>
<td>S-layer attached to peptidoglycan layer</td>
</tr>
<tr>
<td></td>
<td>Cytoplasmal lipid membrane</td>
</tr>
<tr>
<td></td>
<td>Teichio acids and lipoids present (adhesion)</td>
</tr>
</tbody>
</table>

A commonly studied Gram-positive bacterium in relation to uranium bioreduction is the *Clostridium* species which is the one of the major bacterium involved in the reduction of uranium acidic mine pit water. Reduction of uranyl(VI) by *Clostridium* has been observed at pH 3.0, 4.2 and 5.2, and the rate of reduction was reported to be faster at pH 4.2. Interestingly, the concentration of nitrate in solution does not have an effect on the rate of uranium reduction by *Clostridium* species, which is a notable difference to the Gram-negative bacteria *Geobacter* and *Shewanella*.

*Clostridium* sp. have also been shown to reduce uranyl(VI)-citrate complexes without dissociation of the complex. UV-vis spectroscopy showed a change in the oxidation state of the uranium centre from uranyl(VI) to uranium(IV) but EXAFS showed that the oxygen atoms involved in the uranium-citrate bonds were not affected and that the axial uranium-oxygen bonds were converted to single bonds as a consequence of the bacterially mediated electron transfer. The uranium(IV)-citrate species remained in solution which could cause issues during bio-remediation of contaminated land if this
process was observed in place of the formation of the insoluble uranium(IV) species uraninite.

Another species of Gram-positive bacteria that has been studied for uranium bioreduction belongs to the *Desulfitobacterium* genus. Bioreduction studies with this strain have shown that they are able to reduce uranyl(VI) to uranium(IV) in less than 10 days.\(^{61}\) However, the product formed was not identified as uraninite, but either as uranyl(VI) ions individually bound to solid phase ligands or molecular uranium(IV) minerals such as CaU(PO\(_4\))\(_2\).\(^{61}\)

The multitude of research into the ability of different bacteria to reduce uranium has provided many insights into the chemical and biological behaviour that occurs during the various reduction processes. However, much of this research is carried out on microcosm scales with very specific laboratory conditions and for the bio-remediation process to be fully explored *in situ* testing at contaminated sites is essential.

### 1.8 *In situ* Experiments

*In situ* testing at contaminated sites is important to confirm that the results observed during lab scale reductions are applicable to larger natural situations and to assess the feasibility of bioreduction as a successful remediation tool. The Rifle Integrated Field Research site in Colorado has been used to investigate *in situ* bioreduction experiments. From 1924-1958 the site was a uranium and vanadium mill and residual contamination is still present.\(^{62}\)

Analysis of the groundwater at the Rifle site in Colorado determined that *Geobacter* species was the predominant species during the stimulation of iron reduction by acetate injections into the subsurface, and that they were also responsible for the uranium reduction that occurred in the groundwater.\(^{62}\) *In situ* experiments concluded that as long as acetate was continuously available then the bioreduction would be unimpeded. The two summer experiments, which were referred to as ‘Winchester’ in 2007 and ‘Big Rusty’ in 2008 demonstrated that by adding acetate as an electron donor, the bioactivity of the naturally growing bacteria was increased which reduced uranium concentrations to below the U.S Environmental Protection Agency’s drinking water standard of 0.126μM.\(^{62}\)
Another *in situ* test was carried out at the Rifle site in 2010 to investigate the rate of the *in situ* reduction and the role of a bicarbonate injection.\textsuperscript{63} It was hypothesised that the bicarbonate would enhance the uranyl(VI) bioreduction rates by mobilising any uranium(VI) that had incorporated into sub surface minerals. The results showed that the rate was increased in hot spots that were found around the injection site, and that these rates were comparable to laboratory scale results.\textsuperscript{63} However, outside of these hot spots the rate of uranyl(VI) bioreduction was found to be much slower.

The Oak Ridge site in Tennessee has also been used for *in situ* testing where, biweekly two day additions of ethanol were added to contaminated land to stimulate the growth of denitrifying, iron-reducing and sulphate-reducing bacteria.\textsuperscript{64} The concentrations of uranium detected decreased to low levels, and upon addition of sulphite to remove any dissolved oxygen, the concentrations fell below the US. Environmental Protection Agency’s maximum contaminant limit for drinking water. However, once sulphite additions stopped, the concentration of uranium(VI) rapidly increased near the injection well due to the presence of dissolved oxygen.\textsuperscript{64}

These *in situ* experiments show that the bioreduction of uranium can be performed on a large scale, but that many factors may affect the remobilisation of uranium and therefore constant monitoring of the sites is essential.

### 1.9 Luminescence Spectroscopy

The principal spectroscopic techniques generally used for bioreduction experiments are X-ray based techniques such as X-ray Absorption Near Edge Structure (XANES) or Extended X-ray Absorption Fine Structure (EXAFS), which can involve a lot of time and sample preparation. Luminescence spectroscopy on the other hand is a highly sensitive alternative and/or complementary spectroscopic technique that can be carried out *in situ* and includes both fluorescence and phosphorescence.\textsuperscript{16}

When a photon of light is absorbed by a molecule, an electron is excited from a ground state to an excited state. From this excited state, the electron can non-radiatively decay to the ground state or it can decay and emit a photon – this process is fluorescence and is generally quite short lived as there has been no change in the spin of the electron. The electron in the excited state may also undergo intersystem crossing to an excited triplet state – a process which involves changing the spin of the electron. The electron can then decay to the ground state from the triplet state in a process known as phosphorescence.
which is longer lived due to the change in the electron spin. These processes are summarised in Figure 8 below.

**Figure 8**: Jablonski diagram of luminescence emission pathways in an organic or inorganic molecule.

### 1.10 Luminescence Spectroscopy of Uranium

Luminescence spectroscopy has been widely used to study actinide chemistry with uranium being one of the most studied actinides in this area. Each oxidation state of uranium has a distinctive optical profile, thereby allowing, in principle, differentiation between the various oxidation states of uranium by spectroscopy and microscopy. However, this has yet to be demonstrated for uranyl(VI) and uranium(IV). The electronic transition of uranyl(VI) is generally accepted as a formally Laporte forbidden ligand-to-metal charge transfer transition involving promotion of an electron from an ‘yl’ bonding oxygen orbital to a non-bonding uranium orbital, resulting in absorption centred around 420 nm. Relaxation back to the ground state results in formal phosphorescence centred around ca. 520 nm with up to 6 vibrational hot bands observed due to strong coupling of the Raman active total symmetric uranyl bond stretching vibration ($\nu_1$) with the long lived excited state. This results in a characteristic vibrationally resolved emission spectrum and long emissive lifetimes (typically microseconds) that has long been used to optically fingerprint uranyl(VI) species.
Figure 9: Typical absorption (blue trace), emission (green trace, excitation at 420 nm) and excitation (red trace, emission at 520 nm) spectra of uranyl(VI).

Meinrath introduced the potential for using luminescence spectroscopy for environmental uranium samples, showing a large variety of uranium species including $\text{UO}_2^{2+}$ and $\text{UO}_2^\text{OH}^+$ found across a wide range of pH values. It was also concluded that uranyl(VI) carbonate species such as $\text{UO}_2\text{CO}_3$, $\text{UO}_2(\text{CO}_3)_3^{4+}$ and $\text{UO}_2(\text{CO}_3)_2^{2-}$ play an important role within the environment.\textsuperscript{65} Carbonate ligands are known to quench uranyl(VI) emission at room temperature through non-radiative decay and therefore this may pose problems when studying uranyl(VI) emission, particularly at neutral and high pH values.\textsuperscript{66} It was also noted that the lifetimes of the different hydrated $\text{UO}_2^{2+}$ species were not very characteristic and may therefore be difficult to interpret.\textsuperscript{65}

Research into uranyl(VI) luminescence is mostly focused on compounds containing organic ligands, as these are easier to detect since speciation is greatly simplified. Redmond \textit{et al.}, reported the luminescence spectra of various $[\text{UO}_2\text{Cl}_2(L)_2]$ compounds, where $L = \text{thf, Ph}_3\text{PO, Ph}_3\text{PNH and Ph}_3\text{AsO}$.\textsuperscript{67} By varying the ligand, $L$, it was possible to correlate any observed changes in optical properties to this change in coordination experienced by the uranium centre following direct excitation into the uranyl(VI) Ligand-to-Metal Charge Transfer (LMCT) absorption band at ca. 420 nm. They were able to show that the uranyl(VI) emission experienced a significant red-shift as the sigma donating strength of the donor ligand in the equatorial coordination belt increased and this was accompanied by an increase in luminescence lifetime. The characteristic emission profile for uranyl(VI) in an organic ligand complex was also observed by Zucchi \textit{et al.}, who complexed uranyl nitrate with 2,2'-bipyrimidine (BPM) and found
emission in the green region between 465 and 575 nm following excitation into the BPM ligand.\textsuperscript{68}

However, research into the luminescence of aqueous uranyl(VI) species is much less common. Studies of aqueous uranyl(VI) species at low pH show the characteristic vibrational fine structure expected for the +VI oxidation state following excitation at 266 or 355 nm.\textsuperscript{69} Bioaccumulation studies at low pH with \textit{Euglena mutabilis} cells show that up to 95 % of uranium in solution is bioaccumulated by the cells and a shift to higher wavelengths is evident in the uranyl(VI) spectra.\textsuperscript{70} This suggests a change in coordination environment of the uranyl(VI) species when in contact with the bacteria, which was attributed to reactions with carboxylic and or phosphate groups associated with the cells.\textsuperscript{70}

Differences in the emission profile obtained from luminescence spectroscopy can be used to distinguish between the different oxidation states. Uranyl(V) emission was first reported by Steudtner \textit{et al.,} who photo-reduced uranyl(VI) and reported the luminescence of the uranyl(V) species in perchlorate solutions at pH 2.4 following pulsed laser excitation at 255 or 408 nm.\textsuperscript{71} A deconvoluted fluorescence emission spectrum of the sample, shown in figure 10, shows a broad uranyl(V) peak at 440 nm, which is noticeably different from the uranyl(VI) spectrum observed at 510 nm. The lifetime data obtained was mono-exponential, indicating one species was present with a lifetime of $1.1 \pm 0.021 \mu\text{s}$ which is slightly longer than the comparable uranyl(VI) lifetime of $0.9 \pm 0.1 \mu\text{s}$ (Figure 11).\textsuperscript{71}

Further data to support the profile of uranyl(V) emission was reported with the investigation of a uranyl(V) carbonate species.\textsuperscript{72} The samples were excited at either 255 or 408 nm and a similar broad spectrum can be observed between 380 and 440 nm with maxima at 404.7 nm (excitation 255 nm) and 413.3 nm (excitation 408 nm).\textsuperscript{72} The inherent instability of the uranyl(V) species means it is very difficult to obtain accurate uranyl(V) emission, especially in aqueous environments without the use of bulky supporting ligands which can stabilise the uranium centre.\textsuperscript{73}
Uranium(IV) luminescence has also been investigated to elucidate spectroscopic differences between the oxidation states of uranium. In single crystals of LiYF$_4$:U$^{4+}$ emission bands observed at 262, 282, 304, 328 and 334 nm were assigned as transfer transitions between the $^3$F$_2$ 5f$^6$6d$^1$ excited state configuration and the $^3$H$_4$ 5f$^2$ ground state. The luminescence lifetimes of the transitions were determined as 17 ns at both 300 and 77 K. Kirishima et al., also observed uranium(IV) emission in perchloric acid at pH 1 after excitation at 245 nm, resulting in 10 peaks at 525, 409, 394, 345, 338, 335, 320, 318, 291 and 289 nm. These are assigned as the radiative relaxation processes from the $^1$S$_0$ 5f$^2$ excited state to various ground states. Interestingly, it has recently been demonstrated that in non-aqueous solutions, when the ligands around the U(IV) ion are no longer H$_2$O, the $^3$F$_2$ excited state of the 5f$^6$6d$^1$ is lower in energy that the $^1$S$_0$ 5f$^2$ excited state, resulting in three broader emission bands (Figure 12) of charge transfer character in U(IV) halide and polyaminocarboxylate complexes (compared to sharper f-f transitions that resemble lanthanide(III) emission profiles as observed by Kirishima). Again, the luminescence lifetimes at ambient temperature and in frozen solution at 77 K are similar and multi-exponential ranging from ca. 2-20 ns.
Figure 11: A typical emission spectrum of UCl$_4$ in THF at room temperature following excitation at 331 nm; the transitions are due to emission from the $^3$F$_2$ excited state to the $^3$F$_3$ state (365 nm), the $^1$G$_4$/$^1$D$_2$ states (421 nm) and the $^3$P$_1$ state (500 nm). Other transitions to the $^3$H$_4$ ground state, the $^3$F$_2$, $^3$H$_5$, and $^3$F$_3$ states are calculated (and observed) to occur at 251, 290, 292 and 334 nm respectively.$^{76}$

1.11 Two-Photon Spectroscopy

Two-photon excitation is the simultaneous absorption of two photons of light of the same energy resulting in population of an excited state. This can result in the emission of light of higher energy than the excitation wavelength used. This has many advantages over one-photon excitation; the absorbance of two photons only occurs in a small focal volume giving localised excitation. The excitation photons are lower in energy than that required for one photon absorbance and this allows deeper tissue penetration and reduced photo-damage.$^{78}$

1.12 Two-Photon Spectroscopy of Uranium

Two-photon spectroscopy is now becoming widely used in the life sciences for background free optical imaging of cellular species. The technique relies on the simultaneous absorption of two photons of low energy, usually near infra-red (nIR), which act to populate a real higher energy excited state, resulting in the same emission as observed in a one photon process, normally visible. The main advantages are that the use of nIR light avoids tissue damage and the coincidental excitation of endogenous biomolecules, whose fluorescence can compete with that of the dye. Moreover, the use of nIR light enables much deeper tissue and material penetration (typically ca. 500 µm) as
most materials are optically transparent at these wavelengths, and the selection rules of two-photon spectroscopy means that three dimensional images can be recorded, so a confocal set up is not required. Uranium species such as Cs$_2$UO$_2$Cl$_4$ and CsUO$_2$(NO$_3$)$_3$ have been studied in the solid state using two-photon spectroscopy with a visible laser (532 nm).\cite{79} The data provided from this technique gave spectra that were much more highly resolved than the one photon data and allowed calculations on the electronic excited states to be carried out, identifying 14 excited states for the Cs$_2$UO$_2$Cl$_4$ species and 9 excited states for the CsUO$_2$(NO$_3$)$_3$ species.\cite{80} Two-photon spectroscopy requires high-powered femtosecond pulsed lasers and therefore two-photon studies with uranium are limited to these two examples.

### 1.13 Fluorescence Microscopy

Fluorescence microscopy is the combination of luminescence techniques and microscopy resulting in fluorescent images of the samples analysed. Time Resolved Emission image Microscopy (TREM) which can be seen as Fluorescence and Phosphorescence Lifetime Image Microscopy (FLIM and PLIM), can be used on any timescale allowing sub-micron resolution of biological systems, as well as allowing the lifetimes of species present to be spatially resolved and used to provide information about the immediate molecular environment.\cite{81}

One example for the use of FLIM has been used to study the RNA:DNA ratios within microorganisms by measuring microbial activity. Different levels of activity were determined with the use of a nucleic acid-specific fluorochrome, taking advantage of the fact that in actively growing bacteria there is a higher concentration of RNA than in inactive bacteria.\cite{82} The advantage of FLIM in this situation means the extraction of RNA:DNA was not necessary and it can therefore be applied as an *in situ* technique and can easily be extended to environmental microbial communities.\cite{82}

Two-photon FLIM can also be exploited, for example in the study of protein-protein interactions within bacteria such as *E. coli*.\cite{83} Fluorescence lifetime images of the bacteria expressing different proteins were obtained by mapping the different lifetimes obtained across the surface of the bacteria.\cite{83} As can be seen in figure 12, the colour coded scale bar is used to map the fluorescence lifetimes and the lifetimes obtained range from 1.3 ns (blue) to 2.1 ns (red).
Figure 12: Two-photon fluorescence intensity and fluorescence lifetime images of E. coli expressing various proteins, taken from reference 83.

1.14 Fluorescence Microscopy of Uranium

Uranium has been briefly studied with fluorescence microscopy, for example the toxicity of uranium towards bacteria was studied by Grossman et al. With the use of a fluorophore either, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) or 4,6-diamidino-2-phenylindole (DAPI), the metabolic activity of a biofilm exposed to uranium was studied. The increased consumption of oxygen and increased metabolic activity of the bacteria, visualised using fluorescence microscopy, suggested that toxic heavy metals such as uranium have a strong effect on the metabolism of microbes.

The direct use of the emissive properties of uranyl(VI) in fluorescence microscopy has also been used to study the interaction between biofilms and uranium. Fluorescent uranyl(V) and uranyl(VI) particles were observed and characterised in vivo for a growing biofilm. The particles were simultaneously observed in the biofilm and identified with confocal laser scanning microscopy (CLSM) showing a wavelength range of 415-475 nm indicative of uranyl(V) and 480-460 indicative of uranyl(VI) (Figure 13). The use of fluorescence microscopy allowed the identification of the separate species, which may have been lost in bulk analysis techniques.

Figure 13: Observation of uranyl(VI) in a multispecies biofilm by confocal scanning laser microscopy in an x-z cross section, taken from reference 86.
There is a need to develop optical imaging of uranyl(VI) with the direct use of the uranyl(VI) emission rather than the use of fluorophores as this would simplify the systems being analysed and the data collected.

1.15 References


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2.0 Chapter 2: Aims

The aim of this work is two-fold; first to use a variety of optical spectroscopic techniques to investigate the bioreduction of uranium by 2 different strains of bacteria; *Geobacter sulfurreducens* and *Shewanella oneidensis* to further the understanding of the whole bioreduction process, including interactions at the cell surface of the bacteria. The second aim is to demonstrate the applicability of these spectroscopic techniques which have rarely been used for imaging uranium and have not been utilised to study the interaction between uranium and bacterial cells.

Initially, luminescence spectroscopy will be used to investigate the emission of uranyl(VI) during a bioreduction, using a well-established bioreduction system, such as that with *Geobacter sulfurreducens*. It is hoped that by sampling more frequently and measuring the uranyl(VI) concentration throughout the bioreduction process, the role of any intermediate uranium species, such as uranyl(V) can be established. Further work with various mutants of the *Shewanella oneidensis* bacteria will be carried out in an attempt to identify the roles of various surface bound species, such as c-type cytochromes, and to investigate the uranium(IV) product formed during the reduction. Fluorescence microscopy will allow a deeper investigation into these surface bound species, and hopefully contribute towards the understanding of their role within the bioreduction process.

These optical spectroscopy techniques have never been used to study the bioreduction of uranium before and through these studies the applicability and usefulness of these techniques such as fluorescence and phosphorescence lifetime image microscopy (FLIM and PLIM) as well as two-photon spectroscopy and microscopy will be established, furthering their use in this field and in the wider scientific community. Samples will be analysed using a range of fluorescence microscopy techniques to investigate the feasibility of using the inherent uranyl(VI) fluorescence, avoiding the use of organic dyes which can complicate speciation within samples.

With a deeper understanding of the bioreduction process including the interaction between the bacteria and the uranium, the bioreduction of uranium could become a viable technique for the remediation of uranium contaminated land. It is hoped that this work will contribute towards the deeper understanding and highlight the benefits of using luminescence spectroscopy and fluorescence microscopy in this field.
3.0 Paper 1: Fluorescence spectroscopy and microscopy as tools for monitoring redox transformations of uranium in biological systems.

The bioreduction of uranium is a fairly well established process with many different strains of bacteria being investigated for their potential use for in situ land remediation techniques. One of the simplest strains to culture and investigate is the Geobacter sulfurreducens strain as it requires simple growth medium and has been demonstrated to reduce uranyl(VI) to uranium(IV) many times in the literature.

Luminescence spectroscopy is a well-established technique for studying the various oxidation states of uranium in organic systems. Less research has been conducted into the use of luminescence spectroscopy in biologically relevant conditions. The aim of this initial research was to test the applicability of luminescence spectroscopy for monitoring the bioreduction of uranium. Fluorescence microscopy is an emerging field which holds a lot of potential for the study of in situ bioreduction, especially with uranium as there is no need for dyes in the system as the uranyl(VI) species can provide the necessary fluorescence for the imaging.

This chapter explains the results of the experiments conducted with Geobacter sulfurreducens. Luminescence spectroscopy was carried out at both room and low temperature over a wide range of conditions to establish the best method for investigating this process. Fluorescence microscopy was utilised in an attempt to visualise the process on a micron scale and to explore the potential use of fluorescence microscopy in this field.

All the experimental data was collected and analysed by the author, including bacteria growth, uranium luminescence experiments and fluorescence microscopy work carried out at the Rutherford Appleton Laboratories (RAL) in Oxford. The paper was written by the author, with editing help from M.B. Andrews, who along with A.N. Swinburne helped with the development of experiments and knowledge of the field. S.W. Botchway and A. Ward provided help during the experimental set-up of the fluorescence microscopy data used at RAL. J.R. Lloyd (microbiology) and L.S. Natraj (spectroscopy) were the academic supervisors for this piece of work.
Showcasing research from Louise Natrajan’s laboratory, School of Chemistry, University of Manchester, United Kingdom in collaboration with the Lasers for Science Facility, Rutherford Appleton Laboratory, Oxford, United Kingdom.

Fluorescence spectroscopy and microscopy as tools for monitoring redox transformations of uranium in biological systems

The article describes, for the first time, how a combination of luminescence spectroscopy, microscopy and lifetime image mapping can offer new insights into the bioreduction of Geobacter sulfurreducens with uranyl (UVO22+). Geobacter s. is a common bacterium found in sub surface soils and is being actively researched for its ability to enzymatically reduce environmentally mobile toxic radionuclides, here uranium, to their insoluble and therefore immobile counterparts, for the remediation and clean up of nuclear wastes.

As featured in:
See Louise S. Natrajan et al., Chem. Sci., 2015, 6, 5133.

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Fluorescence spectroscopy and microscopy as tools for monitoring redox transformations of uranium in biological systems†

Debbie L. Jones,a Michael B. Andrews,a Adam N. Swinburne,a Stanley W. Botchway,b Andrew D. Ward,b Jonathan R. Lloydc and Louise S. Natrajan*ad

We report a study of redox reactions of uranium in model conditions using luminescence spectroscopy, which with its ease and wide availability has the potential to offer new insights into a bioremediation strategy of particular interest – the enzymatic reduction of U(VI)2+ by bacteria such as Geobacter sulfurreducens. The inherent luminescent properties of U(VI)2+ have been combined with confocal fluorescence microscopy techniques and lifetime image mapping to report directly on uranium concentration, localisation and oxidation state in cellular systems during uranium bioreduction, suggesting that localisation of uranyl species on the cell membrane surface plays an important role and that extracellular biogenic features form alongside uranyl sorbed cellular species during early stages of the bioreduction. The use of confocal microscopy in tandem with lifetime image mapping offers both improved temporal and spatial resolution (nanoseconds to microseconds and sub-micron respectively) than more conventional X-ray based techniques and offers the potential to image redox reactions occurring in situ. Together, these techniques provide an excellent and sensitive probe to assess the coordination environment of uranium during bioreduction processes that are currently being considered for remediation strategies of redox active radionuclides present in contaminated land.

Introduction

The oxidation state of any metal is of vital importance when considering their impact on biological and environmental systems. Oxidation state determines the coordination geometry, bond strength, and Lewis acidity (and therefore the tendency to undergo oligomerisation) and underpins speciation of the metal ion. Additionally, many metal ions are involved in oxidative stress, which arises from the formation of reactive oxygen or nitrogen species and has been implicated in a wide range of diseases.1 Optical spectroscopy provides a convenient, non-destructive, and direct method of monitoring the electronic structure of metal ions in complex systems. Luminescence spectroscopy in particular combines high sensitivity, broad applicability, and low cost, making it an attractive option for studying metal oxidation states over more technologically demanding and/or restricted techniques such as X-ray diffraction, X-ray absorption and electron paramagnetic resonance techniques (XRD, XAS, EPR) which often require extensive sample preparation. It has also been combined with optical microscopy to form fluorescence microscopy, confocal microscopy, and, more recently, two-photon excitation microscopy2 and super resolution microscopy,3 which can provide both spatial and temporal data on a variety of chemical species in a biological setting.4,5

Luminescence spectroscopy is an ideal technique for the study of uranium speciation. Since the development of nuclear power and weapons, containment breaches at all stages of the fuel cycle have led to elevated levels of uranium in the environment.6 Although there are concerns associated with its radioactivity and long half-life, the hazards of uranium are primarily due to its chemical toxicity.7 The dominant form of uranium under oxic, environmental conditions is U(VI)2+, a potent nephrotoxin. The uranyl cation is also very soluble and super resolving techniques focus largely on the reduction of U(VI)2+ to the less soluble U(IV) cation.8 The inherent

†Electronic supplementary information (ESI) available: Full experimental details. Luminescence decays and fits, PXRD patterns, scintillation experiment results, additional experimental details and results for control experiments including comparative data with Escherichia coli. Anomalous data points resulting from cosmic rays were removed from steady state spectra obtained, and unaltered spectra are included in the ESI. The histogram showing the distribution of lifetime measurements in Fig. 3 shows only pixels with greater than 1000 counts, the full distribution can be found in the ESI. See DOI: 10.1039/c5sc00661a

References

photophysical properties of the uranyl cation, arising from partially forbidden charge transfer transitions from oxo-based molecular orbitals to non-bonding, unoccupied f-orbitals, provide a convenient means of monitoring uranyl concentration, speciation, and movement without the need of additional imaging agents (such as dye probes). Despite recent time-resolved laser fluorescence spectroscopy (TRLFS) studies into the bioaccumulation of uranium, the use of optical techniques has been largely underutilised in favour of assay-based and scintillation techniques, to provide concentration data, and X-ray-based techniques such as EXAFS and EDX (coupled with electron microscopy), to provide structural data. Here, we report the use of luminescence spectroscopy in combination with confocal fluorescence and phosphorescence microscopy and lifetime image mapping in the study of a process of great interest to the remediation of uranium in the environment – the bioreduction of U\(^{VI}\) to insoluble U\(^{IV}\)-based mineral-type structures by endogenous bacterial populations.

### Results and discussion

**Luminescence spectroscopy**

The bacterium chosen for this study, *Geobacter sulfurreducens*, is a Gram negative bacterium that is ubiquitous in subsurface soils. *Geobacter sulfurreducens* is well known to enzymatically reduce U\(^{VI}\) under anaerobic conditions and was grown according to literature precedent. This reduction process has previously been studied by assay-based techniques to yield concentration data and EXAFS to give structural data, however many questions still remain on the exact enzymatic mechanisms responsible for the reduction process and despite its sensitivity and greater spatial resolution, luminescence spectroscopy remains underutilised in this field. Under the conditions required for bioreduction to occur (30 mM NaHCO\(_3\), 5 mM UO\(_2\)(CH\(_3\)CO\(_2\))\(_2\)) the emission lifetime of the system at 525 nm was signifi-
cantly more well-resolved (Fig. 1), with the emissive species in this system; it does however suggest that if the interaction of U\(^{VI}\) with the cells is maintained at this temperature it does not significantly contribute to the fluorescent properties of the system. Indeed, at this temperature, the structural integrity of the cellular structure of the bacteria is likely to be compromised. A control study with a uranyl acetate solution shows the same steady-state spectrum and emission lifetime (ESI), demonstrating that the emissive species in the presence of *Geobacter sulfurreducens* is not a uranyl acetate complex. Uranil carbonate species are known to be emissive under cryogenic conditions, showing significant variation in both the energy of the vibronic bands and the emission lifetimes. Comparison of the peak values and lifetime measurements with literature reports for uranyl carbonate and hydroxide species did not allow identification of the emissive species in this system; it does however suggest that if

<table>
<thead>
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<th>Solution</th>
<th>Temp/K</th>
<th>Emission lifetime/μs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM UO(_2)(CH(_3)CO(_2))(_2), 30 mM NaHCO(_3)</td>
<td>293</td>
<td>2.21 ± 0.13 (51)</td>
</tr>
<tr>
<td>5 mM UO(_2)(CH(_3)CO(_2))(_2), 30 mM NaHCO(_3), <em>Geobacter sulfurreducens</em></td>
<td>293</td>
<td>7.06 ± 0.31 (49)</td>
</tr>
<tr>
<td>5 mM UO(_2)(CH(_3)CO(_2))(_2), 30 mM NaHCO(_3), <em>Geobacter sulfurreducens</em></td>
<td>77</td>
<td>8.84 ± 0.46 (61)</td>
</tr>
<tr>
<td>5 mM UO(_2)(CH(_3)CO(_2))(_2), 30 mM NaHCO(_3), <em>Geobacter sulfurreducens</em></td>
<td>77</td>
<td>20.98 ± 1.17 (39)</td>
</tr>
<tr>
<td>5 mM UO(_2)(CH(_3)CO(_2))(_2), 30 mM NaHCO(_3), <em>Geobacter sulfurreducens</em></td>
<td>77</td>
<td>1198.20 ± 9.02</td>
</tr>
<tr>
<td>5 mM UO(_2)(CH(_3)CO(_2))(_2), 30 mM NaHCO(_3), <em>Geobacter sulfurreducens</em></td>
<td>77</td>
<td>1125.62 ± 12.63</td>
</tr>
</tbody>
</table>
multiple species were present, this would be visible in both the steady-state and time-resolved spectra. The simplified speciation enabled the concentration of uranyl to be monitored and quantified over the course of the bioreduction experiment (see ESI† for calibration experiments). At regular time points an aliquot of the solution was removed from the reaction and frozen in liquid nitrogen, an emission spectrum was then obtained under a standardised instrumental set-up (see ESI† for further details). Over eight hours the emission intensity showed a general decrease, and after one day the solution was completely non-emissive, suggesting that microbial reduction had proceeded to completion. This interpretation was supported by scintillation techniques, (ESI†) and the formation of the 

U⁷⁺ compound uranium (UO₂), the primary product of microbial reduction of UV⁴⁺ with Geobacter sulfurreducens,²⁴-²⁶ confirmed by powder X-ray diffraction (Fig. S12, ESI†). Control studies carried on dead cells (ESI, Fig. S14†) showed only a slight decrease in fluorescence intensity over the course of 24 hours. This is in good agreement with literature reports and demonstrates that the process is enzymatic and not simply dependant on biogenic reductants which may be released in the absence of metabolic activity. It also demonstrates that the observed decrease in fluorescence intensity is unlikely to be simply due to sorption of uranium onto cellular material, as the extent of sorption by dead cellular material is known to be generally equal to or greater than the extent of sorption by living biomass.²⁷,²⁸

The ease and speed with which photoluminescence spectroscopy can be carried out allowed this process to be studied at higher temporal resolution than previously available,⁴⁴ revealing an unusual feature during the early stages of the bioreduction. Although the emission intensity of U⁷⁺⁴⁻ underwent a general decrease over eight hours, the rate of this decline was not consistent (Fig. 2, right). Instead, the process was seen to occur in several stages, in which an initial sharp decrease in emission intensity was followed by partial increase in intensity. This distinctive ‘saw tooth’ pattern was obtained reproducibly and cannot be attributed to instrumental or experimental error. There are several possible processes that may account for this phenomenon. Although the reoxidation of U⁷⁺ to U⁴⁺ has been observed under nominally reducing conditions, this process occurs over much greater timescales (up to 500 days) and in more ‘geomimetic’ conditions in which nitrate, Fe⁴⁺ and Mn⁴⁺ may act as terminal electron acceptors.²⁹-³⁰ An alternative explanation may rest in changes in the uranyl speciation. If the most emissive U⁷⁺⁴⁻ was preferentially reduced by the bacteria³¹,³² a sharp decrease in emission intensity resulting from bioreduction may be followed by a gradual increase as the system re-equilibrates. This explanation, however, does not take into account the simplified uranium speciation profile at 77 K at which these results were obtained. A more likely explanation, it seems, is that the delayed rise in fluorescence intensity is due to the disproportionation of an unstable U⁷⁺ intermediate which is non-fluorescent in the 450–600 nm spectral window employed. As enzymatic U⁷⁺⁴⁻ reduction, a first order reaction with regards to [U⁷⁺⁴⁻] occurs meaning the concentration of U⁷⁺⁴⁻ and rate of reaction steadily decrease. At the same time, the concentration of U⁵⁺⁴⁻ increases and the rate of disproportionation, a second-order reaction, will increase quadratically. This may account for the fluctuations in the concentration, and hence emission intensity, of U⁷⁺⁴⁻ as it is gradually reduced to U⁴⁺ via an unstable U⁵⁺⁴⁻ intermediate. Moreover, liquid scintillation counting experiments over the same time period show a linear decrease in total soluble uranium content, giving further weight to this argument (ESI, Fig. S13†). Indeed, direct and indirect evidence has previously pointed to the existence of a U⁵⁺⁴⁻ intermediate,⁸ suggesting that the enzymatic reduction of U⁷⁺⁴⁻ by Geobacter sulfurreducens is in fact a one electron reduction, followed by disproportionation of U⁵⁺⁴⁻ to form U⁴⁺ and U⁷⁺⁴⁻, and it appears that emission spectroscopy supports this conclusion.

Fluorescence microscopy

Having shown that the inherent emission of the U⁷⁺⁴⁻ ion provides a suitable handle for monitoring uranium oxidation state during enzymatic reduction by Geobacter sulfurreducens, the same process was then studied by fluorescence microscopy at sub-micron spatial resolution in order to probe the locality/ (bio) distribution of uranyl both within bacterial communities

![Graph 1](image1.png)

**Fig. 1** Emission spectra of the uranyl cation in a carbonate buffer solution (30 mmol) in the presence of Geobacter sulfurreducens at room temperature (solid line) and 77 K (dotted line). (λex = 420 nm). Aliquot obtained immediately after introduction of UO₂(CH₃COO)₂ (5 mmol) to microcosm and brief agitation.

![Graph 2](image2.png)

**Fig. 2** The decrease in the intensity of uranyl emission over time in the steady-state emission spectrum of anaerobic uranyl solutions containing Geobacter sulfurreducens (λex = 420 nm, 77 K).
and with isolated single bacterium. The bioreduction reaction was carried out as described previously, with the only exception being that aliquots were placed directly onto a glass slide for observation by microscopy, which was carried out at room temperature. The microscope was equipped with a 405 nm diode laser; while this is not the peak excitation wavelength it does excite into the same uranyl absorption band as used in the spectroscopic experiments. Additionally a CCD-based spectrometer enabled the collection of steady-state spectra on objects observed under the microscope, full instrumental details can be found in the ESI.† Immediately after the introduction of bacteria to the uranyl solution, relatively large extracellular masses (ca. 7–15 microns in size) that displayed intense fluorescence in the green-channel were clearly visible alongside the bacterial cells.

Fluorescence lifetime image mapping (FLIM) and phosphorescence lifetime image mapping (PLIM) revealed that these features displayed long-lived emission, up to 130 µs (Fig. 3, left) suggesting that this was due to uranyl fluorescence rather than biological autofluorescence. A steady-state spectrum obtained directly from one such extracellular mass confirmed this, showing characteristic, well-resolved uranyl emission (Fig. 3, right) along with weak auto fluorescence. The bright-field image (Fig. 4, top left) and in the absence of uranium showed very little autofluorescence. When uranyl acetate was added, the cells became clearly visible by FLIM, (Fig. 4, top right) having experienced a significant enhancement in fluorescence. The bright field and FLIM images are clearly superimposable (Fig. 4, bottom left) indicating that the emissive uranium is associated with the bacterial cells; no significant emission was recorded in the PLIM window. As observed in the solution luminescence spectroscopy experiments, the spectrum obtained from the bacteria showed a broad, unresolved peak centred at 515 nm consistent with the presence of multiple emissive species, (Fig. 4, bottom right) along with marginal autofluorescence in the red region (ESI, Fig. S7†). Over several hours this fluorescence diminished, coinciding with the conversion of extracellular, UVO2.2+–containing material and the formation of nanoparticulate UO2. In contrast to the extracellular material, the UVO2.2+ fluorescence from the surface of the cells was extremely short-lived (ca. 300–800 ps, ESI Fig. S17†). This is not unexpected, as the reduction potential of the uranyl cation is considerably increased on photoexcitation and transitory reduction to a UO2.2+ species is considered to be a significant quenching mechanism. In fact, the short fluorescent lifetime may principally be due to the presence of species involved in the electron-transport chain in which UVO2.2+ acts as the terminal electron acceptor. This is further supported by control experiments carried out on a Escherichia coli, which is known to sorb but not reduce UVO2.2+. These studies demonstrated an enhancement in fluorescence intensity of E.

Having demonstrated that FLIM and PLIM are suitable methods for studying the bioreduction of uranium by *Geobacter sulfurreducens*, attention turned to the cells themselves. The rod-shaped, micron-sized cells were clearly visible in the bright-field image (Fig. 4, top left) and in the absence of uranium showed very little autofluorescence. When uranyl acetate was added, the cells became clearly visible by FLIM, (Fig. 4, top right) having experienced a significant enhancement in fluorescence. The bright field and FLIM images are clearly superimposable (Fig. 4, bottom left) indicating that the emissive uranium is associated with the bacterial cells; no significant emission was recorded in the PLIM window. As observed in the solution luminescence spectroscopy experiments, the spectrum obtained from the bacteria showed a broad, unresolved peak centred at 515 nm consistent with the presence of multiple emissive species, (Fig. 4, bottom right) along with marginal autofluorescence in the red region (ESI, Fig. S7†). Over several hours this fluorescence diminished, coinciding with the conversion of extracellular, UVO2.2+–containing material and the formation of nanoparticulate UO2. In contrast to the extracellular material, the UVO2.2+ fluorescence from the surface of the cells was extremely short-lived (ca. 300–800 ps, ESI Fig. S17†). This is not unexpected, as the reduction potential of the uranyl cation is considerably increased on photoexcitation and transitory reduction to a UO2.2+ species is considered to be a significant quenching mechanism. In fact, the short fluorescent lifetime may principally be due to the presence of species involved in the electron-transport chain in which UVO2.2+ acts as the terminal electron acceptor. This is further supported by control experiments carried out on a *Escherichia coli*, which is known to sorb but not reduce UVO2.2+. These studies demonstrated an enhancement in fluorescence intensity of *E.*

**Fig. 3** Phosphorescence lifetime image map focusing on a representative extracellular feature in samples taken immediately after the introduction of *Geobacter sulfurreducens* to uranyl acetate solution (left), a histogram showing the distribution of lifetime measurements (centre), and a representative, uranyl spectrum from one such extracellular feature (right) ($\lambda_{ex} = 405$ nm, room temperature).

**Fig. 4** Bright-field microscopy image (top left), FLIM image (top right), Overlaid bright-field and FLIM image (bottom left) of *Geobacter sulfurreducens* and representative spectrum taken from one such bacterium ($\lambda_{ex} = 405$ nm, room temperature).
coli cells in the presence of uranyl acetate but significantly longer fluorescence lifetimes (ESI Fig. S15 and S16).

Furthermore, altering the excitation focus in the z direction (±2 μm), resulted in a slight decrease in emission intensity upon passing through the Geobacter sulfurreducens cells. Although the spatial resolution of the FLIM and PLIM images do not enable the precise location of the uranium within the bacterial cells to be determined, these data may indicate that the uranium is principally associated with the cell walls and/or the outer cell membranes rather than incorporated into the cell. However, since the reduction in lifetime was not observed by spectroscopy in the bulk samples on short timescales (ns), it is likely that the surface bound species make up a very minor component of the total uranyl speciation. Further, gene deletion experiments alongside proteomic studies have suggested that both outer membrane and periplasmic c-type cytochromes play a central role in extracellular electron transport in the bioreduction of U(VI) with Geobacter sulfurreducens. 18

Conclusions

In summary, we have demonstrated the utility of readily available, facile luminescence spectroscopy in studying the bioreduction of U(VI) by Geobacter sulfurreducens over a 24 hour time period. The speed at which a spectrum can be obtained, and the minimal sample preparation involved has allowed the progress of the reaction to be studied in situ, importantly, at shorter time-increments than previously possible, providing supporting evidence towards the role of a UO₂ intermediate. We have also demonstrated for the first time the viability of using the inherent fluorescent properties of the U(VI) to report directly on the localisation of uranium by fluorescence and phosphorescence lifetime imaging with the absence of uranium, however, on the addition of uranyl acetate there is an immediate increase in emission associated with the surface of the cells, this suggests a previously unconsidered step in the enzymatic reduction process – the absorption of U(VI). The extremely short luminescent lifetime suggests that the uranyl is interacting with electron-rich species, potentially electron transfer proteins localised towards the periphery of the outer membrane of the Gram-negative bacterial cell, such as cytochromes (including c-type) which have been implicated in the reduction of uranium27 and those that are known to bind U(III). 28–30 The identification of extracellular material has suggested the applicability of this technique to the study of other bioremediation techniques such as biosorption, bioaccumulation and biominalisation. This approach could provide new insights into the fate of uranium in more complex microbial–mineral–ground water systems, which are currently poorly understood. Further work will also aim at incorporating two-photon microscopy as a potential means of providing additional spectroscopy and spatial data.

Acknowledgements

We are grateful to Professor David Collison for helpful discussions and the EPSRC for funding a Nuclear First DTC studentship [DJ] (EP/G004846/1), a Career Acceleration Fellowship [LN], postdoctoral funding [MA, AN], the Leverhulme Trust for additional postdoctoral funding [MA, AN] (RL-2012-072) and a research Leadership award [LN], the NERC and the STFC for funding direct access to the Laser for Science Facilities at Rutherford Appleton Laboratories. We are also grateful to the STFC funded Environmental Radiation Network (Env-Rad-Net) for facilitating this work.

Notes and references

Electronic Supporting Information (ESI) for Fluorescence spectroscopy and microscopy as tool for monitoring redox transformations of uranium in biological systems

Debbie L. Jones, Michael B. Andrews, Adam N. Swinburne, Stanley W. Botchway, Andrew Ward, Jonathan R. Lloyd, and Louise S. Natrajan*

Sample Preparation
All manipulations of cells were carried out under an atmosphere of N₂. Late-log-phase cultures were harvested by centrifugation and washed twice in NaHCO₃ buffer (30 mM, pH 7, degassed with N₂-CO₂ 80:20 mix). Aliquots of the washed cell suspensions were added to a final concentration of 0.5 mg mL⁻¹ dry weight biomass, by use of a syringe fitted with a needle, to anaerobic bottles sealed with butyl rubber stoppers containing uranyl(VI) acetate (5mM) in NaHCO₃ buffer (30 mM, pH 7) creating the microcosm for the experiment. No electron donor was added as the acetate from the uranyl acetate acts as the electron donor in this case. No further nutrients were added to prevent cell growth from interfering with the experiments.

Samples were removed from the microcosm every hour, by use of a syringe fitted with a needle and filtered into a young’s tap cuvette, a low temperature EPR tube or placed onto a slide and sealed with a cover slip, for room temperature luminescence, low temperature luminescence and microscopy studies respectively.

Fluorescence Studies
Steady state emission spectra were recorded on an Edinburgh Instrument FP920 Phosphorescence Lifetime Spectrometer equipped with a 5 watt microsecond pulsed xenon flash lamp and a red sensitive photomultiplier in peltier (air cooled) housing, (Hamamatsu R928P). Lifetime data were recorded following 420 nm excitation with a 2 Watt xenon flash lamp (Edinburgh Instruments), using multichannel scaling. Lifetimes were obtained by tail fit on the data obtained, and quality of fit judged by minimization of reduced chi-squared and residuals squared.

For consistency and in order to ensure reproducibility steady-state luminescence experiments were carried out using identical instrumental settings and all data reported are an average of at least 3 runs:

<table>
<thead>
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<th>Instrument Setting</th>
<th>Value</th>
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<td>Step</td>
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</tr>
<tr>
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<tr>
<td>Emission Monochromator Bandwidth</td>
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</tbody>
</table>
**Fig. S1.** Steady state emission profile of room temperature solutions of uranyl acetate (5 mmol) in sodium carbonate buffer (30 mmol) in the absence (green line) and presence of *Geobacter sulfurreducens* (purple line) and following freezing and defrosting (black line) as well as the scattering caused by OD600 0.1 *Geobacter sulfurreducens* in carbonate buffer in the absence of uranium (room temperature – red line, 77K blue line).

**Fig. S2.** Steady state emission profile of 77 K solution of uranyl acetate (5 mmol) in sodium carbonate buffer (30 mmol) in the absence (solid line) and presence of *Geobacter sulfurreducens* (dashed line). (λ<sub>ex</sub> = 420 nm)
Fig. S3. Kinetic trace of uranyl acetate in buffer solution at room temperature (top); black squares represent recorded data, black line represents polyexponential fit. Residuals of fit also plotted (bottom). ($\lambda_{ex} = 420$ nm, $\lambda_{em} = 503$ nm)
Fig. S4. Luminescent lifetime of uranyl acetate and *Geobacter sulfurreducens* in buffer solution at room temperature (top); Black squares represent recorded data, black line represents polyexponential fit. Residuals of fit also plotted (bottom). ($\lambda_{ex} = 420$ nm, $\lambda_{em} = 503$ nm)

$\tau_1 = 8.8432 \mu$s (61.45 %)
$\tau_2 = 20.9843 \mu$s (38.55 %)
$\chi^2 = 0.935$
Fig. S5. Luminescent lifetime of uranyl acetate in buffer solution at 77 K (top); black squares represent recorded data, black line represents polyexponential fit. Residuals of fit also plotted (bottom). ($\lambda_{ex} = 420$ nm, $\lambda_{em} = 503$ nm)
Fig. S6. Luminescent lifetime of uranyl acetate with *Geobacter sulfurreducens* in buffer solution at 77 K (top); black squares represent recorded data, black line represents polyexponential fit. Residuals of fit also plotted (bottom). ($\lambda_{ex} = 420$ nm, $\lambda_{em} = 503$ nm)

**Uranyl nitrate control study**

In order to gain further insight into the speciation of uranyl solutions at 77 K a control study was carried out using the same concentration of $\text{UO}_2(\text{NO}_3)_2$ in carbonate buffer. At 77 K the steady state and luminescence lifetime spectra were identical to those obtained from the uranyl acetate solution.
Fig. S7. Steady-state emission spectrum of UO$_2$(NO$_3$)$_2$·6H$_2$O (5 mM) and NaHCO$_3$ (30 mM) in frozen aqueous solution (77 K). ($\lambda_{\text{ex}} = 420$ nm)

Fig. S8. Luminescent lifetime of uranyl nitrate in buffer solution at 77 K (top); black squares represent recorded data, black line represents polyexponential fit. Residuals of fit also plotted (bottom). ($\lambda_{\text{ex}} = 420$ nm, $\lambda_{\text{em}} = 503$ nm)
**Uranyl concentration vs. Emission intensity control study**

In order to ensure that the emission intensity of the frozen solutions (77 K) represented the uranyl concentration and there were no inner filter effects operative, a calibration study was carried out using varying concentrations of uranyl acetate in the carbonate buffer solution. It was found that there was a linear relationship between uranyl concentration and emission intensity.

![Graph showing the linear relationship between uranyl concentration and emission intensity.](image)

\[
y = 92499x + 14052 \\
R^2 = 0.9837
\]

**Fig. S9.** Luminescence Intensity of the main 503 nm emission band plotted against uranyl concentration in carbonate buffer solution (30 mM NaHCO\(_3\)). (\(\lambda_{ex} = 420\) nm, \(\lambda_{em} = 503\) nm)

**Microscopy**

The 1 photon-excitation FLIM-PLIM is a commercial system from Becker and Hickl with a 405 nm pulsed laser system. Fluorescence lifetime images were obtained using a modified confocal microscopy apparatus, constructed in the Central Laser Facility, which has a Nikon eC1 scanhead. Laser light was focused through a x60 water immersion objective with an NA of 1.2 on an inverted Nikon microscope (TE2000-U). Emission was collected without descanning, by-passing the scanning system, and passed through a bandpass filter (BG39, Comar). The scan was operated in the normal mode and line, frame and pixel clock signals were generated and synchronized with an external fast micro-channel plate photomultiplier tube (Hamamatsu R3809U) used as the detector. These were linked via a time-correlated single photon counting (TCSPC) PC module SPC830 (Becker and Hickl). The set-up provided instrument quantum efficiencies of more than 50% with single photon detection capabilities. Steady-state grey-scale multiphoton images (8 bit, up to 256 x 256 pixels) are produced by binning all decay photons as a single channel. Emission lifetime images were obtained by analysing the decay at individual pixels using a single or double exponential curve fitting following some modification to the standard Becker-Hickl SPCimage analysis software (B&H SPCimage 2.94) to allow analysis of the microsecond decay domain. A thresholding function within the analysis software ensured that non-correlating photons leading to background noise arriving at the detector were not included in the analysis. The lifetime image data are presented without further image processing. Steady state spectra were recorded using an Ocean Optics USB2000+ spectrometer.
**Fig. S10.** Example of characteristic uranyl spectrum obtained from extracellular material following excitation at 405 nm. High intensity peaks (full height 5000 – 20000 A.U.) due to interference by cosmic rays and removed from Fig. 3 in main text.

**Fig. S11.** Example of broad uranyl (centred at 510 nm) and biological autofluorescence (centred at 670 nm) spectrum of *Geobacter sulfurreducens* following excitation at 405 nm. High intensity peak (full height 7800 A.U.) due to interference by cosmic rays.

**Powder X-ray Diffraction (PXRD)**

To identify the black precipitate produced by the bioreduction experiments an aliquot taken at the end of the experiment was centrifuged to separate insoluble material. This was then dried and applied directly to the sample holder and was analysed via powder X-Ray Diffraction (pXRD; Bruker D8 Advance using Cu-Kα radiation and EVA 14 analytical software).
**Fig. S12.** PXRD pattern of black crystalline species formed during the bio-reduction of uranium. Black trace is the recorded pattern, red trace is calculated pattern.

**Liquid Scintillation Experiments**

For analysis of the samples using liquid scintillation counting, a 100 μl sample was mixed with scintillant (Scintisafe 3, Fisher Scientific) and 1 mL HCl prior to counting on a LSC (Quantulus, PerkinElmer) instrument. All samples were run in triplicate.

**Table S2.** Average concentration of soluble uranium at various points during the bio-reduction process.

<table>
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<tr>
<th>Time (hrs)</th>
<th>Avg concentration of uranium kBq mL⁻¹</th>
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Fig. S13 Luminescence Intensity of the main 503 nm emission band plotted against liquid scintillation concentration values for the first 4 hours of the bio-reduction. Values normalised against reading taken at 0 hours.

**Control Experiments**

Experiments were conducted as described above using bacteria which were autoclaved at 125°C to confirm that the reduction of uranium was occurring solely due to enzymatic processes mediated by living bacteria and not due to any other (trace) reducing species. As Fig. S14 (below) shows, the concentration of uranium decreased only slightly across the 8 hour period indicating that the metabolically active bacteria play a vital role in the reduction of the uranium.
**Fig. S14.** Plot depicting luminescence intensity of uranium with autoclaved dead cells of *Geobacter sulfurreducens*; data normalised according to intensity at 0 hours.

Further control experiments were carried out using *Escherichia coli*, which was grown as described for *Geobacter sulfurreducens*. Samples with and without uranyl acetate were studied.
**Fig. S15.** Brightfield (top left) and FLIM (top right) microscopy images of *e. coli* in the absence of uranyl acetate (scale bar applies to both) as well as a representative spectrum taken from the surface of a cell (bottom left) and histogram showing fluorescence lifetime distribution (bottom right). All fluorescence results obtained using 405 nm excitation.
Fig. S16. Brightfield (top left) and FLIM (top right) microscopy images of *E. coli* in the presence of 5 mmol uranyl acetate (scale bar applies to both) as well as a representative spectrum taken from the surface of a cell (bottom left) and histogram showing fluorescence lifetime distribution (bottom right). All fluorescence results obtained using 405 nm excitation.
Additional FLIM/PLIM data

**Fig. S17.** Histogram showing full distribution of emission lifetime measurements from one uranyl-containing extracellular feature

**Fig. S18.** FLIM image of *Geobacter sulfurreducens* alongside one uranyl-containing extracellular feature following excitation at 405 nm.
**Fig. S19.** Histogram showing full distribution of emission lifetime measurements from the FLIM image in Fig S.18 (405 nm excitation).
4.0 Paper 2: Multiphoton Imaging of Spatial Distribution, Coordination and Redox Environment of a key Radionuclide under Model Biogeochemical Conditions.

Intention to submit to Nature.

In the previous chapter, one-photon microscopy demonstrated an interaction between uranyl(VI) and the surface of the bacteria during the reduction process. Two-photon microscopy can be used to explore this interaction further, with better spatial resolution available allowing for a larger variation in lifetimes to be observed.

During two-photon excitation two low energy photons are absorbed via an induced, or ‘virtual’ energy level to reach the same excited states as one-photon absorption. The quadratic power dependence allows control of excitation in the z-dimension and using near infrared excitation allows for deeper tissue penetration and reduced photo damage. When combined with microscopy, this could allow improved resolution in fluorescence microscopy imaging, allowing for a more detailed understanding of the interactions occurring on a cellular level.

As two-photon microscopy has never been studied with uranyl(VI) before, standardised experiments were conducted to determine the applicability of the process. These demonstrate the potential for two-photon spectroscopy and microscopy to be utilised when studying uranium interactions with various biological systems, without the use of molecular dyes which could interfere with the system.

The data surrounding the *Geobacter sulfurreducens* results was collected and analysed by the author, and this section of the paper was also written by the author. The remaining data was collected and analysed by M.B. Andrews who wrote the rest of the paper. S.W. Botchway and A. Ward were the collaborators from the Rutherford Appleton Laboratories (RAL) in Oxford. J.R. Lloyd (microbiology) and L.S. Natrajan (spectroscopy) were the academic supervisors for this piece of work.
Multiphoton Imaging of Uranium under Biogeochemically Relevant Conditions


The interaction of uranium with biological materials is of interest not just because of its chemical and radiological toxicity but also from a remediation standpoint. The inherent photoluminescent properties of uranyl (UO$_2^{2+}$) provide a convenient handle to monitor its location and chemical speciation. The use of luminescence spectroscopy and microscopy is hindered, however, by the need to excite uranyl high energy UV-light. Here we show that two-photon absorption can provide an alternative mode of excitation. Spectroscopic studies have been used to determine fundamental photophysical properties while fluorescence- and phosphorescence- lifetime imaging microscopy has shown to be capable of observing the sorption of uranyl by various forms of microbial life in vivo. We anticipate that this could enable the development of depth-profiling of biological tissue and real-time flow experiments, providing significant insight into uranyl behaviour in biogeochemically relevant conditions.

Introduction

At many sites around the world artificially elevated levels of radionuclides have been observed in the environment, arising from nuclear weapons tests and containment breaches at various stages of the nuclear fuel cycle.\(^1\) While the radiological effects of spent fuel present a severe threat to the global ecology the chemical toxicity of uranium, the main component of nuclear waste, also poses a significant risk.\(^2\) While no known organism has evolved to use uranium, several species of anaerobic dissimilatory bacteria are able to incorporate uranium into their respiratory cycle as the terminal electron acceptor. This process results in the reduction of the soluble uranyl cation (UO$_2^{2+}$), the dominant form of uranium in oxic environments, to insoluble U(IV) minerals and has been proposed as a potential remediation strategy.\(^3\) Bioreduction has typically been studied by liquid scintillation or assay-based techniques, which are limited to giving purely quantitative data on the concentration of uranium in solution. Further work has been carried out on these systems using EXAFS, to provide data on the coordination geometry of uranium, and various electron microscopy techniques, which can probe uranium distribution on the cellular level. These techniques, however, are not widely available and require significant sample preparation. Many questions remain to be answered on the mechanisms of potential bioremediation techniques, such as the nature of cell-surface species involved, as well as the role of uranium speciation and the influence of coordinating and competing ions in solution. The range of techniques used to study these processes must be expanded to allow real-time, in vivo monitoring in systems that better emulate environmental and geological conditions.

As one of the more easily handled actinides uranium has been extensively studied with the linear, triatomic uranyl unit in particular being representative of a bonding motif found throughout the earlier actinides. The inherent photoluminescence of the uranyl cation makes a
convenient handle for studying both solution and solid-state chemistry. This vibronically structured emission, centred around 520 nm, arises from transitions from molecular orbitals of partially oxygen p-orbital character to non-bonding f-orbitals. Both the spectral profile and the lifetime, which ranges up to the millisecond regime, are responsive to the equatorial coordination and chemical speciation. Our previous work showed that it was possible to study this emission using Fluorescence and Phosphorescence Lifetime Imaging Microscopy (FLIM and PLIM, respectively) in which photoluminescence indicates the location of an analyte on a cellular level while the steady-state profile and lifetime provide data on the local environment of the analyte at a molecular level.\(^4\) This involved resonant, one-photon excitation of uranyl, relying on high-energy ultraviolet light which is detrimental to biological systems. Additionally, one-photon FLIM and PLIM suffers from high absorption and autofluorescence. If dynamic processes are to be studied in vivo as potential bioremediation strategies these drawbacks must be overcome by developing non-destructive analytical and imaging techniques. One such alternative approach is non-resonant, or multiphoton, excitation. The most common example of MPE is two-photon excitation (2PE), which involves the simultaneous absorption of two photons via a transitory virtual state.\(^5\) Relaxation back to the ground state may then occur, releasing a single photon of higher energy than the incident radiation. As excitation relies on red or near-infrared (NIR) light it is less damaging to biological tissue, has deeper penetration depths and can result in lower autofluorescence. Additionally, due to the quadratic dependence of excitation on photon flux, 2PE allows control of excitation in the z-dimension, an advantage for microscopic investigations of spatially-complex samples. These benefits have led to the development and application of dyes for use in two-photon microscopy, aimed at imaging particular cellular structures and processes. In spite of this, the only studies that have been carried out on the two-photon excitation of uranyl have used crystalline samples, often under cryogenic (liquid helium) conditions.\(^6\)\(^-\)\(^8\)

Here we demonstrate that uranyl emission can be induced by 2PE in the NIR at room temperature and in amorphous phases. We then image uranyl with 2PE microscopy in yeast, shedding new light on the biosorption process, and in bacterial systems, allowing direct visualisation of the redox chemistry on the cell surface.

**Results**

**Spectroscopic Studies**

In order to determine whether the 2PE emission of uranyl species could be used in microscopy the emissive properties of various uranyl containing species were studied in solution at ambient temperature, using a femtosecond Ti:Sapphire laser with an operating range of 690 – 1040 nm. A range of ligand types and coordination geometries were selected and care was taken to ensure that the solvents used as well as the concentrations of uranyl, counterions, and ligands would result in simple speciation profiles. The species studied by Barker and coworkers,\(^6\)\(^,\)\(^7\) [UO\(_2\)Cl\(_4\)]\(^2-\) and [UO\(_2\)(NO\(_3\))]\(^3-\), were used as model systems and [UO\(_2\)(H\(_2\)O)]\(^3+\), [UO\(_2\)(CH\(_3\)CO\(_2\))]\(^3-\), and [UO\(_2\)(dpa)]\(^2-\) were chosen as representative of biogeochemically relevant species. Solutions were prepared according to literature precedent\(^9\)\(^-\)\(^10\) and the one-photon excitation (1PE) spectra were obtained to identify the peak excitation wavelengths for each species (Figure 1, solid line). 2PE experiments were then carried out at double these
wavelengths, and each species showed strong characteristic uranyl luminescence (Figure 1, dashed line). The emission profiles that resulted from 1PE and 2PE were very similar, with any subtle differences likely arising from the different spectrometer resolution. In each case a log-log plot of emission intensity against laser power demonstrated quadratic power dependence (SI Figures S2, S5, S9, S13, S17), verifying that the emission observed was the result of 2PE. Two-photon excitation spectra were obtained between 730 and 1040 nm by adjusting the laser and correcting for changes in laser power. Although the broad emission profile of the pulsed laser limits spectral resolution vibronic structure was visible in each case.

Two-photon excitation spectra were obtained between 730 and 1040 nm by adjusting the laser and correcting for changes in laser power. Although the broad emission profile of the pulsed laser limits spectral resolution vibronic structure was visible in each case.

The ability of a molecule to undergo 2PE is quantified as its two-photon cross section, which is reported in Goeppert-Mayer (GM) units with 1 GM = 10^{-50} cm^4 s photon^{-1}. These can be measured directly or by comparison to a dye standard, as was carried out here with Coumarin 485 chosen as the standard due to the proximity of the emission maximum with that of the uranyl species. For each species two-photon cross sections were obtained over a range of wavelengths to produce low-resolution spectra that approximately corresponded with the two-photon excitation spectra, shown in table 1 (full graphical data in SI Figures S1, S4, S8, S12, S16). This represents the first time that the direct (non-sensitized) two-photon cross section of an f-block metal has been recorded. Two-photon dyes have values ranging from single digits to the thousands. Compared to this, the uranyl species studied here possess low two-photon cross sections. This is unsurprising, as the organic molecules for which these values are usually

Figure 1 | One- and two-photon spectra of uranyl complexes. A, Emission spectra of [UO_2Cl_4]^{2-} in acetonitrile (10 mM) following one-photon (420 nm) and two-photon (840 nm) excitation. B, One- and two-photon excitation spectra of [UO_2Cl_4]^{2-} in acetonitrile (10 mM), emission intensity taken at 523 nm. C, One- and two-photon excitation spectra of [UO_2(NO_3)_3]^{3-} in acetonitrile (10 mM), emission intensity taken at 507 nm. D, One- and two-photon excitation spectra of [UO_2(H_2O)_5]^{2+} in 0.1 M HNO_3 (10 mM), emission intensity taken at 510 nm. E, One- and two-photon excitation spectra of [UO_2(CH_3CO_2)_3]^{2-} in 1 M CH_3CO_2H (10 mM), emission intensity taken at 511 nm. F, One- and two-photon excitation spectra of [UO_2(dpa)_2]^{2-} in water (2 mM), emission intensity taken at 5 nm. All intensities in arbitrary units.
reported are designed to show structural features such as extended π-conjugated system and Donor-π-Acceptor-π-Donor arrangements that are known to result in high cross sections.

Table 1 | Photophysical constants of uranyl complexes used in this study. [UO$_2$Cl$_4$]$^{2-}$ in acetonitrile (10 mM), lifetime taken at 523 nm. [UO$_2$(NO$_3$)$_3$] in acetonitrile (10 mM), lifetime taken at 507 nm. [UO$_2$(H$_2$O)$_5$]$^{2+}$ in 0.1 M HNO$_3$ (10 mM), lifetime taken at 510 nm. [UO$_2$(CH$_3$CO$_2$)$_3$] in 1 M CH$_3$CO$_2$H (10 mM), lifetime taken at 511 nm. [UO$_2$(dpa)$_2$]$^{2-}$ in water (2 mM), lifetime taken at 5 nm. All lifetimes measured using 405 nm diode laser.

<table>
<thead>
<tr>
<th>Uranyl Species</th>
<th>Extinction Coefficient (M$^{-1}$cm$^{-1}$)</th>
<th>Emission Lifetime (μs)</th>
<th>Quantum Yield</th>
<th>Peak Photon Section (GM)</th>
<th>Two-Photon Cross Section (GM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[UO$_2$Cl$_4$]$^{2-}$</td>
<td>37.0</td>
<td>34.8</td>
<td>3.6 %</td>
<td>6.40 x 10$^3$</td>
<td></td>
</tr>
<tr>
<td>[UO$_2$(NO$_3$)$_3$]</td>
<td>26.4</td>
<td>3.3</td>
<td>1.7 %</td>
<td>1.42 x 10$^3$</td>
<td></td>
</tr>
<tr>
<td>[UO$_2$(H$_2$O)$_5$]$^{2+}$</td>
<td>12.3</td>
<td>1.9</td>
<td>4.2 %</td>
<td>1.28 x 10$^4$</td>
<td></td>
</tr>
<tr>
<td>[UO$_2$(CH$_3$CO$_2$)$_3$]</td>
<td>12.7</td>
<td>0.14</td>
<td>1.2 %</td>
<td>2.57 x 10$^4$</td>
<td></td>
</tr>
<tr>
<td>[UO$_2$(dpa)$_2$]$^{2-}$</td>
<td>29.5</td>
<td>8.5</td>
<td>14.3 %</td>
<td>5.90 x 10$^4$</td>
<td></td>
</tr>
</tbody>
</table>

Microscopic Studies

Despite the unpromising cross sections of the uranyl species, experiments were carried out to discern whether 2PE microscopy could be used to image uranium in microbial systems. Due to its wide availability and a wealth of literature precedent$^{12}$ Brewer’s yeast (Saccharomyces cerevisiae) was chosen as a model system. In the absence of uranium, after being applied directly to a microscope slide without fixation, S. cerevisiae were observed as aggregates of multiple cells. Excitation at 405 nm resulted in intense autofluorescence which typically displayed a bi-exponential decay with a short-lived component of approximately 300 ps and a longer-lived component of 1200-1500 ps. PLIM also showed very weak phosphorescence with a lifetime centred at 300 ns. For initial studies into uranyl biosorption yeast samples were exposed to a concentrated solution of uranyl nitrate (0.1 M, 10 mg yeast/10 ml, unadjusted pH ~ 2.5) as described by Popa and coworkers.$^{13}$ FLIM showed intense, short-lived emission indistinguishable from the uranyl-free control however PLIM now showed long-lived emission centred at 1900 ns. The emission was, however, fairly weak. Raising the pH (by addition of NaOH) resulted in increasingly intense phosphorescence, with little change in lifetime. While the variations in the photophysical properties of uranyl complexes (table 1) can result in significant variations in brightness, the similarity in the lifetimes observed at different pH suggest that instead of changes in speciation the increased intensity at higher pH is due to greater amounts of uranyl being sorbed. This is consistent with previous studies which show that pH is the most important factor in uranyl biosorption, with minimal accumulation occurring at low (2.5) and near-neutral pH (>6) and maximum accumulation taking place in mildly acidic conditions (pH 4-5).$^{13-15}$
The ability to study uranyl adsorption by two-photon excitation microscopy was tested by adjusting the pH using a carbonate source (NaHCO$_3$) to better emulate the uranyl speciation in groundwater. Carbonate has a high binding coefficient and is known to have a rich coordination chemistry with uranyl, with carbonate solubilising uranyl in high pH solutions in which oligomerisation would otherwise occur. Additionally, carbonate is known to quench uranyl emission at room temperature, resulting in weakly emissive complexes with very short luminescent lifetimes. These factors suggest that carbonate may preferentially bind to and solubilise uranyl, preventing biosorption while quenching emission and making imaging uranyl adsorption by luminescence microscopy difficult. Indeed, reduced sorption of uranyl onto clay minerals and some biomass in the presence of carbonate has been observed. In fact, in these systems this was not the case, as PLIM revealed that as the pH is raised with NaHCO$_3$ there is a significant increase in intensity of phosphorescence associated with the yeast cells (Fig. 2, centre and right). While the effect of pH on the accumulation of uranium by yeast has been widely studied to the best of our knowledge the pH has not been adjusted using carbonate and further studies using better analogues for natural conditions are needed.

While the previous examples demonstrate that 2PE FLIM/PLIM can be used to detect the adsorption of uranyl to yeast, the concentrations are not representative of either the majority of sorption studies or the concentrations of uranium found in contaminated sites. In order to assess the viability of this technique to provide further insight on potential bioremediation techniques it was also applied to yeast which had been exposed to much lower concentrations of uranyl (<3 mM), according to the procedure reported previously by Omar and coworkers. Although the wide range of two-photon cross sections and quantum yields of uranyl species (cf. Table 1) prohibit the exact quantification of a lower limit for the detection of uranyl in biological systems by 2PE microscopy, the quantity of uranyl removed from solution by yeast was measured in order to estimate the sensitivity of this technique (SI, Figures S19, S20). In accordance with the results obtained at high uranyl concentration the presence of carbonate did not show a significant adverse effect on the adsorption of uranyl. The yeast samples from
these solutions were determined to have adsorbed approximately 1.5 – 2 mmol uranium per gram of dry biomass. After separation by centrifugation these yeast samples were observed using 2PE microscopy and both showed significantly more phosphorescence than observed in the uranyl-free control sample. The system in which the pH had been adjusted using NaOH showed phosphorescence with lifetimes ranging from 1500 – 3000 ns with a peak at 2500 ns, while the carbonate-containing system showed a broad range of phosphorescent lifetimes with a peak at 11500 ns (SI, Figure S24).

**Uranyl Bioreduction Studies**

To further assess the use of two-photon microscopy in the study of potential bioremediation techniques our earlier work on the enzymatic reduction of uranyl was expanded to incorporate 2PE. *Geobacter sulfurreducens*, a Gram-negative anaerobic strain of bacteria that has long been known to reduce uranyl,\(^\text{18}\) was cultured according to literature procedure, washed, and resuspended in a carbonate buffer. Our previous report showed that in the absence of uranyl very little autofluorescence was detected,\(^4\) however on addition of 5 mM of UO\(_2\)(CH\(_3\)CO\(_2\))\(_2\) two changes were observed – the appearance of extracellular features showing strong, characteristic uranyl emission and a significant increase in fluorescence associated with the bacterial cells.

In accordance with the microscopy studies on uranyl biosorption by yeast cells, excitation of *G. sulfurreducens* in the presence of uranyl with a pulsed, NIR laser (810 nm) resulted in metal-based emission. The images also demonstrated excellent resolution, without the need for an additional pinhole as in confocal microscopy, allowing the visualisation of distinct areas of shorter- and longer-lived emission spread over the cell surface (Figure 3). Control experiments using *Escherichia coli*, a bacterium which is known to absorb but not reduce uranyl\(^\text{19}\) showed an increase in the intensity of fluorescence but consistent lifetimes over the surface of the cell (SI, Figure S25).

![Figure 3 | Two photon microscopy of uranyl-*geobacter*. PLIM intensity view (left) and PLIM lifetime view (right) show cells after exposure to uranyl solution (5 mM), following two-photon excitation. \(\lambda_{ex} = 810\) nm. Scale bar applies to both.](image)

In our previous work the increase in fluorescence intensity and the associated emission spectrum of Geobacter cells demonstrated the sorption of uranyl. The extremely short lifetime of this emission was attributed to the presence of electron donating species localised on the outer membrane of the cell as it is well established that transitory reduction to U(V) is a significant quenching mechanism of uranyl emission. It is possible that even in the absence of light these reducing species are involved in the electron transfer chain in which uranyl acts as
the terminal electron acceptor and that the species are unevenly distributed over the surface of the cell. Previous studies have highlighted the involvement of OmcB50 and OmcS51 cytochromes as playing key roles in electron transfer to Fe(III) and hence potentially other metals including uranyl at the periphery of the Geobacter cell, while the potential orientation of these proteins at the cell surface was discussed in detail by Liu et al.

Conclusions

Taken together, these results demonstrate that characteristic uranyl emission can be used in 2PE microscopy to directly study uranium distribution and molecular environment in cellular systems, without the need for additional dyes. While uranyl species show very low two-photon cross sections, comparable to that of common biological fluorophores, the long-lived uranyl emission is readily observed by PLIM. The ability to map aggregates of biomass and uranyl-containing materials while simultaneously probing the coordination environment of the metal using lifetime imaging has been applied to the biosorption of uranyl by yeast to demonstrate that carbonate does not appear to inhibit binding. This stands in contrast to the sorption of uranyl on clay minerals and suggests that further work needs to be applied to study biosorption in solutions that better emulate groundwater, potentially in concert with other speciation-sensitive techniques such as Raman mapping. While most bacteria are an order of magnitude smaller than the fungal cells used for biosorption the high resolution obtained from two-photon microscopy has shown it to be a potentially useful technique for studying the enzymatic reduction of uranyl to U$^{IV}$ by dissimilatory bacteria such as Geobacter sulfurreducens. In particular, there has been significant debate over the role of species such as outer membrane-bound cytochromes as well as extracellular structures such as pili and other “nanowires” in the enzymatic reduction process. The ability to excite uranyl using NIR light, which can penetrate biological tissues without damaging them, suggests the capability to use this technique to study dynamic bioremediation processes in vivo and over time. The use of 2PE lifetime mapping microscopy as a means of imaging the redox potential of a cell wall, combined with complimentary techniques such as TEM, also has the potential contribute significantly to this field in pure culture and mineral-containing systems.

References


Preparation of Uranyl Solutions for Spectroscopic Measurements

Caution! Whereas the uranium oxynitrate used in this study was prepared from depleted uranium, standard precautions for handling radioactive substances should be observed. Chemicals used were purchased from Sigma-Aldrich or Fisher and used without further purification. Dry Brewer’s Yeast was obtained from MP Biomedicals.

[UO2Cl4]2-
Uranyl tetrachloride was prepared according to a modified version of a literature precedent.\(^1\) Uranyl dichloride and tetrabutylammonium chloride (2 eq.) was dissolved in acetonitrile to produce a 10 mM solution.

[UO2(NO3)3]3-
Uranyl trinitrate was prepared according to literature precedent.\(^2\) Uranyl dinitrate hexahydrate and tetrabutylammonium nitrate (1 eq.) was dissolved in acetonitrile to produce a 10 mM solution.

[UO2(H2O)5]3+
Uranyl pentahydrate was prepared according to literature precedent\(^3\) by preparing a 10 mM solution of [(UO2)(NO3)3].6H2O in 0.1 M HNO3.
[UO₂(CH₃COO)₃]⁺

Uranyl triacetate was prepared according to literature precedent by preparing a 10 mM solution of [(UO₂(NO₃)₃].₆H₂O in 1 M CH₃COOH.

[UO₂(DPA)₂]²⁻ (DPA = dipicolinic acid/2,6-pyridinedicarboxylic acid)

Uranyl dipicolinate was prepared according to literature precedent. [(UO₂(NO₃)₃].₆H₂O (50 mg, 1 eq. 0.1 mmol) was dissolved in deionised water (2 ml) and heated to 90 °C. A separate solution was prepared by dissolving 2,6-pyridinedicarboxylic acid (35 mg, 2 eq. 0.2 mmol) and Na₂CO₃ (21 mg, 2 eq. 0.2 mmol) in deionised water (2 ml) and heating to 90 °C until the evolution of gas had ceased. The 2,6-pyridinedicarboxylate solution was added to the uranyl solution, upon cooling to room temperature bright yellow/green, needle-like crystals of Na₂[UO₂(DPA)₂] formed in near quantitative yield. These were removed by filtration, washed with acetone and dried under vacuum. Na₂[UO₂(DPA)₂] was then dissolved in deionised water to produce a 2 mM solution for spectroscopic studies.

One-Photon Fluorescence Studies

Steady state emission spectra were recorded on an Edinburgh Instrument FP920 Phosphorescence Lifetime Spectrometer equipped with a 450 watt steady state xenon lamp and a red sensitive photomultiplier in peltier (air cooled) housing, (Hamamatsu R928P). Lifetime data were recorded following 405 nm excitation with a picosecond pulsed diode laser (Edinburgh Instruments, model EPL-405) or a 5 watt xenon flash lamp (Edinburgh Instruments) using multichannel scaling. Lifetimes were obtained by tail fit on the data obtained, and quality of fit judged by minimization of reduced chi-squared and residuals squared. Absolute quantum yields were recorded using an Edinburgh Instruments integrating sphere (model FLS980).

Two-Photon Fluorescence Studies

The two-photon emission spectra were determined in the 730 – 1000 nm region using a Ti:sapphire oscillator (Mai Tai HP) generating 100 fs pulses at a rate of 80 MHz. The excitation was focused into the cuvette (10 mm x 10 mm, quartz) through a microscope objective (40x, NA = 0.60, Nikon). The luminescence was detected in epifluorescence mode via a longpass dichroic mirror with a cut-on wavelength 650 nm (Thor Labs, FEL0650). To reduce residual reflected laser light several shortpass filters were used, with cut-off wavelengths of 700, 800 and 900 nm (Thor Labs, FES0700, FES0800 and FES0900). Fluorescence was collected by a compact CCD spectrometer module (Ocean Optics QE65000) and processed using SpectraSuite®. Two-photon excitation spectra were obtained by adjusting the laser wavelength and collecting a spectrum every 5 nm. Intensities were obtained by integrating over the region of peak emission intensity (± 2 nm) and scaled by the square of the laser power at that wavelength.
Two-photon cross sections were obtained using Coumarin 485 standard. Due to the vibronically resolved nature of uranyl emission the cross sections were calculated using the equation described by Mathai et al., adjusting for the difference in emission intensity (integrating over the region of maximum emission, ± 2 nm), concentration, solvent refractive index, and quantum yield and scaled to take into account differences in peak profile.

Microscopy Samples

Solutions of [(UO$_2$)(NO$_3$)$_3$].6H$_2$O in deionised water were prepared according to previously reported studies of uranyl sorption by yeast (0.1 M and 0.5 – 3.0 mM) and the pH was adjusted using NaOH or NaHCO$_3$. Solutions were allowed to equilibrate for 24 hours to ensure no further change in pH or precipitation. Yeast was added according to aforementioned literature reports (weighed by dry mass). After 24 hours the yeast cells were removed from solution by centrifugation (4000 rpm, 15 minutes) and most of the supernatant decanted. Yeast cells were refrigerated at 5 °C in a small residual amount of supernatant, before being applied directly to a microscope slide, placed under a coverslip and sealed using a commercially available nitrocellulose resin (nail varnish).

Bacterial samples were prepared as described previously.

Sorption Studies

After exposure to yeast the lower concentration uranyl solutions were analysed using the Br-PADAP test, according to literature precedent.

Microscopy

The 1 photon-excitation FLIM-PLIM is a commercial system from Becker and Hickl with a 405 nm pulsed laser system. Fluorescence lifetime images were obtained using a modified confocal microscopy apparatus, constructed in the Central Laser Facility, which has a Nikon eC1 scanhead. Laser light was focused through a x60 water immersion objective with an NA of 1.2 on an inverted Nikon microscope (TE2000-U). Emission was collected without descanning, by-passing the scanning system, and passed through a bandpass filter (BG39, Comar). The scan was operated in the normal mode and line, frame and pixel clock signals were generated and synchronized with an external fast micro-channel plate photomultiplier tube (Hamamatsu R3809U) used as the detector. These were linked via a time-correlated single photon counting (TCSPC) PC module SPC830 (Becker and Hickl). The set-up provided instrument quantum efficiencies of more than 50% with single photon detection capabilities. Steady-state grey-scale multiphoton images (8 bit, up to 256 x 256 pixels) are produced by binning all decay photons as a single channel. Emission lifetime images were obtained by analysing the decay at individual pixels using a single or double exponential curve fitting following some modification to the standard Becker-Hickl SPCImage analysis software (B&H SPCImage 2.94) to allow
analysis of the microsecond decay domain. A thresholding function within the analysis software ensured that non-correlating photons leading to background noise arriving at the detector were not included in the analysis. The lifetime image data are presented without further image processing. Steady state spectra were recorded using an Ocean Optics USB2000+ spectrometer.

Figure S1. Absorption, one-photon excitation spectrum ($\lambda_{em} = 520$ nm), two-photon excitation spectrum ($\lambda_{em} = 520$ nm) and two-photon cross section of [UO$_2$Cl$_4$]$^{2-}$ (10 mM in acetonitrile). Excitation spectra arbitrarily scaled.
Figure S2. Log-log plot of intensity of $[\text{UO}_2\text{Cl}_4]^2-$ (10 mM in acetonitrile) emission following two-photon excitation ($\lambda_{\text{em}} = 520 \text{ nm}$, $\lambda_{\text{ex}} = 950$) against laser power.

$$y = 1.8326x - 2.542$$

Figure S3. Luminescence lifetime of $[\text{UO}_2\text{Cl}_4]^2-$ (10 mM in acetonitrile) ($\lambda_{\text{em}} = 520 \text{ nm}$, $\lambda_{\text{ex}} = 420$ nm). $\tau = 34788 \text{ ns}$, Std Dev = 478 ns, $\chi^2 = 0.985$
Figure S4. Absorption, one-photon excitation spectrum ($\lambda_{\text{em}} = 510$ nm), two-photon excitation spectrum ($\lambda_{\text{em}} = 510$ nm) and two-photon cross section of [UO$_2$(NO$_3$)$_3$]$^-$ (10 mM in acetonitrile). Excitation spectra arbitrarily scaled.

Figure S5. Log-log plot of intensity of [UO$_2$(NO$_3$)$_3$]$^-$ (10 mM in acetonitrile) emission following two-photon excitation ($\lambda_{\text{em}} = 510$ nm, $\lambda_{\text{ex}} = 840$ nm) against laser power.

\[
y = 2.0168x - 1.7636
\]
Figure S6. Luminescence lifetime of $[\text{UO}_2\text{(NO}_3\text{)}_3]^{-}$ (10 mM in acetonitrile) ($\lambda_{\text{em}} = 520$ nm, $\lambda_{\text{em}} = 340$ nm). $\tau = 3302$ ns, Std Dev = 47.2 ns, $\chi^2 = 1.051$

Figure S7. Emission Spectra of $[\text{UO}_2\text{(H}_2\text{O})_5]^{2+}$ (10 mM in 0.1 M HNO$_3$) following one-photon (405 nm) and two-photon (810 nm) excitation.
Figure S8. Absorption, one-photon excitation spectrum ($\lambda_{em} = 510$ nm), two-photon excitation spectrum ($\lambda_{em} = 510$ nm) and two-photon cross section of [UO$_2$(H$_2$O)$_5$]$^{2+}$ (10 mM in 0.1 M HNO$_3$). Excitation spectra arbitrarily scaled.

Figure S9. Log-log plot of intensity of [UO$_2$(H$_2$O)$_5$]$^{2+}$ (10 mM in 0.1 M HNO$_3$) emission following two-photon excitation against laser power ($\lambda_{em} = 510$ nm, $\lambda_{ex} = 840$ nm).
Figure S10. Luminescence lifetime of \([\text{UO}_2(\text{H}_2\text{O})_5]^{2+}\) (10 mM in 0.1 M HNO\(_3\)) \((\lambda_{em} = 510 \text{ nm}, \lambda_{ex} = 405 \text{ nm})\). \(\tau = 1880 \text{ ns, Std Dev = 11.5 ns, } \chi^2 = 1.132\)

Figure S11. Emission Spectra of \([\text{UO}_2(\text{CH}_3\text{COO})_3]^-\) (10 mM in 1 M CH\(_3\)COOH) following one-photon (410 nm) and two-photon (820 nm) excitation.
Figure S12. Absorption, one-photon excitation spectrum ($\lambda_{em} = 510$ nm), two-photon excitation spectrum ($\lambda_{em} = 510$ nm) and two-photon cross section of $[\text{UO}_2(\text{CH}_3\text{COO})_3]^{-}$ (10 mM in 1 M CH$_3$COOH). Excitation spectra arbitrarily scaled.

Figure S13. Log-log plot of intensity of $[\text{UO}_2(\text{CH}_3\text{COO})_3]^{-}$ (10 mM in 1 M CH$_3$COOH) emission following two-photon excitation against laser power ($\lambda_{em} = 510$ nm, $\lambda_{ex} = 840$ nm).
Figure S14. Luminescence lifetime of [UO$_2$(CH$_3$COO)$_3$]$^-$ (10 mM in 1 M CH$_3$COOH) ($\lambda_{\text{em}} = 510$ nm, $\lambda_{\text{ex}} = 405$ nm). $\tau = 142$ ns, Std Dev = 1.94 ns, $\chi^2 = 1.192$

Figure S15. Emission Spectra of [UO$_2$DPA$_2$]$^2-$ (2 mM in water) following one-photon (405 nm) and two-photon (810 nm) excitation.
Figure S16. Absorption, one-photon excitation spectrum ($\lambda_{em} = 510$ nm), two-photon excitation spectrum ($\lambda_{em} = 510$ nm) and two-photon cross section of $[\text{UO}_2\text{DPA}_2]^2$ (2 mM in water). Excitation spectra arbitrarily scaled.

Figure S17. Log-log plot of intensity of $[\text{UO}_2\text{DPA}_2]^2$ (2 mM in water) emission following two-photon excitation against laser power. ($\lambda_{em} = 510$ nm, $\lambda_{ex} = 810$ nm).
Figure S18. Luminescence lifetime of [UO$_2$DPA$_2$]$^-$ (2 mM in water) ($\lambda_{em} = 510$ nm, $\lambda_{ex} = 405$ nm).
$\tau = 8530$ ns, Std Dev = 36.7 ns, $\chi^2 = 1.128$

Figure S19. Uranyl sorbed from 0.5 mM solution by Brewer’s Yeast when pH is adjusted with hydroxide or carbonate source. Solubility limited pH range of NaOH solutions to $\leq 6$. 
Figure S20. Uranyl sorbed from 3.0 mM solution by Brewer’s Yeast when pH is adjusted with hydroxide or carbonate source. Solubility limited pH range of NaOH solutions to $\leq 5$.

Figure S21. Steady-state emission spectrum obtained from yeast used in microscopy study.
Figure S22. Luminescence lifetime of a suspension of yeast cells in deionised water ($\lambda_{em} = 510$ nm, $\lambda_{ex} = 405$ nm). $\tau_1 = 8.55$ ns, Std Dev = 0.15 ns, 39 %, $\tau_2 = 363$ ns, Std Dev = 52.9 ns, 34 %, $\tau_3 = 23.08$ ns, Std Dev = 0.93 ns, 27 %, $\chi^2 = 1.602$.

Figure S23. PLIM intensity and lifetime view of yeast following two-photon excitation (810 nm). Pixels binned ten times to obtain intensity required to fit lifetimes.
Figure S24. Bright-field image, PLIM intensity view and PLIM lifetime view of yeast following exposure to uranyl solution (3 mM), adjusted to pH 5 using NaOH (left) and NaHCO₃ (right).
Figure S25. FLIM lifetime view of *Escherichia coli* cells showing generally uniform fluorescent lifetime across cell surface.
References

5.0 Paper 3: An Investigation into the Role of c-type Cytochromes and Extracellular Flavins in the Bioreduction of Uranyl(VI) Acetate by *Shewanella Oneidensis* using Fluorescence Microscopy.

Intention to submit to Chemical Science.

Following investigations into the bioreduction of uranium by *Geobacter sulfurreducens* which highlighted an interaction between uranium and the cell surface of the bacteria, another Gram-negative bacteria was investigated, *Shewanella oneidensis*. Through the use of mutant strains a deeper investigation into the role of surface bound species was carried out. The four strains used in this chapter were; JG274 – wild type, JG596 – lacking outer membrane (OM) c-type cytochromes, JG1453 – lacking inner membrane (IM) and outer membrane (OM) c-type cytochromes, JG1758 – lacking the ability to secrete extracellular flavins such as flavin mononucleotide (FMN).

The strains lacking in varying amounts of cytochromes were used to further investigate the role of cytochromes within the bioreduction system. It is proposed that they are an essential part of the electron transport chain, and therefore their absence would have an effect on the reduction rate. This was further investigated using fluorescence microscopy to probe specific interactions and species on the surface of the bacteria. Flavins are also hypothesised to be involved in the bioreduction of uranium, and comparisons between the flavin secreting and non-flavin secreting strains may provide insight to the role of flavins within a bioreduction system as well as further enhance our knowledge on the quenching potential of flavins.

All the experimental data was collected and analysed by the author, including bacteria growth, uranium luminescence experiments and fluorescence microscopy work carried out at the Rutherford Appleton Laboratories (RAL) in Oxford. This chapter was also written by the author. M.B. Andrews provided support during the development of experiments and during the analysis of data. S.W. Botchway and A. Ward provided help during the experimental set-up of the fluorescence microscopy data used at RAL. J.R. Lloyd (microbiology) and L.S. Natrajan (spectroscopy) were the academic supervisors for this piece of work.
An investigation into the Role of c-type Cytochromes and Extracellular Flavins in the Bioreduction of Uranyl(VI) Acetate by Shewanella oneidensis using Fluorescence Microscopy.


A range of optical spectroscopic techniques have been used to study the in situ bioreduction of uranyl(VI) acetate, with lactate as the electron donor, over a 24 hour period by the Gram-negative metal reducing bacterium Shewanella oneidensis. The wild type strain was used alongside three deletion mutants; JG 596 – lacking outer membrane (OM) c-type cytochromes, JG 1453 – lacking inner membrane (IM) and outer membrane (OM) c-type cytochromes and JG 1758 – lacking the ability to secrete extracellular flavins such as flavin mononucleotide (FMN). Low temperature emission of the uranyl(VI) species monitored over a 24 hour period indicate, for the first time, a one-electron reduction pathway for the wild type whereas no reduction is observed for the JG596 (lacking OM c-type cytochromes) and JG1453 (lacking IM and OM c-type cytochromes) confirming the critical role for c-type cytochromes in the transfer of electrons to U(VI). One and two-photon fluorescence microscopy shows a variation in lifetimes across the surface of the reducing bacterial cell, suggesting an interaction between the uranyl(VI) and a surface bound redox active species. As this variation is not present in the strains lacking cytochromes, it is hypothesised that the cytochromes are interacting with the uranyl(VI) on the cell surface. These techniques allow the direct visualisation of radioclime-microbe interactions during the bioreduction of U(VI), which can provide mechanistic insight into the mechanisms of in situ bioredmediation of uranium (and potentially other actinides) in microbial cultures and more complex environmental systems.

Introduction

Many sites across the world have been dealing with legacy radioactive contamination issues, resulting in an increased effort to investigate the role of these contaminants within the environment.1 With new nuclear investment such as the proposed Hinkley Point C and Wylfa Newydd nuclear power stations in the UK, comes a responsibility to deal with legacy contamination issues to ensure the safety of generations to come. Moreover, containment breaches at different stages during the fuel cycle, along with weapons testing and accidental spillage, has led to elevated levels of uranium within the environment. Successful remediation of these sites is essential to the future of the nuclear industry.2

The oxidation state of the uranium is of vital importance as this will affect the chemical behaviour of the uranium within the environment. Under oxic conditions the most common oxidation state is the +VI state, which forms uranyl, a linear di-oxo species, UO₂²⁺. This species is water soluble and can migrate within the environment and therefore remediation techniques to reduce it to the less mobile +IV oxidation state, U IV⁴, which can precipitate as the black mineral uraninite or coordinate with biomass as poorly crystalline U(IV), will likely prevent its migration.4 The ability to reduce uranyl(VI) to uranium(IV) is widespread amongst metal-reducing bacteria isolated from the subsurface and other anoxic environments.5 In situ studies at the Rifle site in Colorado have shown that in the presence of acetate as an electron donor, naturally growing bacteria, dominated by Geobacter species, are able to reduce uranium(VI) leading to reduced concentrations of soluble uranium within the subsurface.6 Geobacter species, along with another widely studied bacterial strain in this field, Shewanella, are Gram-negative anaerobic bacteria.7 Gram-negative bacteria have an inner and outer membrane, which in metal-reducers contain an abundance of c-type cytochromes whereas Gram-positive bacteria lack the outer membrane but have a thicker cell wall.8
Shewanella oneidensis MR-1 has been widely studied in this field to investigate the role of cytochromes during the bioreduction process. Organic molecules such as acetate or lactate can act as electron donors which feed the electron transport chain within the bacteria. A total of 41 putative c-type cytochromes have been identified during the genome sequencing of Shewanella oneidensis, many of which include multi-heme-containing proteins that are instrumental in the transfer of electrons within the electron transport chain.

Four cytochromes that are hypothesised to be heavily involved in the electron transport chain of Shewanella oneidensis are MtrA, MtrB, MtrC and OmcA. MtrA serves as an entry point for electron flow, MtrB relays electrons through the periplasm to the outer membrane and MtrC interacts with OmcA forming stable complexes as part of the electron transport chain. Exposure of OmcA on the surface of the cell then allows for interaction with electron acceptors, such as uranyl(VI). Moreover, Shewanella species are known to secrete redox active flavin molecules; while flavin adenine dinucleotide (FAD) is retained within the cell, flavin mononucleotide (FMN) and riboflavin are secreted extracellularly, where FMN can catalytically mediate electron transport pathways during microbial uranyl(VI) reduction. The spectroscopic techniques used to study the reduction of uranium and other metals include X-ray based techniques such as X-ray absorption near edge structure (XANES) or extended X-ray absorption fine structure (EXAFS). These techniques can involve complicated and time consuming sample preparation and data analysis and require mM sample concentrations. We have previously reported that fluorescence spectroscopy and microscopy can be used to probe the speciation and lifetimes of Geobacter sulfurreducens bio-reducing uranyl(VI) samples in situ. By monitoring the concentration of uranyl(VI) over a 24 hour period, a clear increase in the uranyl(VI) concentration, depicted by a ‘saw-tooth pattern’ suggested the formation, followed by the disproportionation, of a uranyl(V) intermediate. Fluorescence microscopy demonstrated, for the first time, a direct interaction between uranyl(VI) in solution and bacterial cells with approximately a 1000 fold decrease in the luminescence lifetime of the uranyl(VI) emission observed upon contact with the surface of the bacteria. This demonstrates the ability of fluorescence microscopy to probe the cell surface interactions of bacteria and uranyl(VI), with the possibility of gaining further insight to the role of outer membrane c-type cytochromes and other biological structures, in electron transfer to U(VI).

Here, we report an investigation into the bioreduction of uranium, from UO_{2}^{2+} to insoluble UO_{2} by Shewanella oneidensis with the use of luminescence spectroscopy and confocal fluorescence microscopy and two-photon fluorescence microscopy combined with lifetime image mapping. The use of deletion mutants of the Shewanella oneidensis MR-1 strain, lacking in outer membrane cytochromes, both inner and outer membrane cytochromes and lacking in the ability to secrete flavin co-factors, has provided a much-needed deeper insight into the role of c-type cytochromes within the bacterial electron transport chain and the effect of extracellular flavins on the rate of the bioreduction process.

**Experimental**

**Maintenance and Growth of organisms**

Four strains of *S. oneidensis*; JG 274 – wild type, JG 596 – lacking outer membrane (OM) cytochromes, JG 1453 – lacking inner membrane (IM) and outer membrane (OM) cytochromes, JG 1758 – lacking the ability to secrete extracellular flavins such as flavin mononucleotide (FMN), were grown at 30°C under aerobic conditions on LB agar plates for 24 hours before being transferred to LB broth liquid medium for a further 24 hours.

Table 1: Summary of the differences between each of the four *Shewanella oneidensis* strains used during this investigation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>JG 274</td>
<td>Wild type, no alterations</td>
</tr>
<tr>
<td>JG 596</td>
<td>Lacking outer membrane cytochromes</td>
</tr>
<tr>
<td>JG 1453</td>
<td>Lacking inner and outer membrane cytochromes</td>
</tr>
<tr>
<td>JG 1758</td>
<td>Lacking the ability to produce flavins</td>
</tr>
</tbody>
</table>

**Resting Cell Experiments**

All manipulations of cells were carried out under an atmosphere of N2. Late-log-phase cultures were harvested by centrifugation and washed twice in NaHCO3 buffer (30 mM, pH 7, degassed with N2-CO2 80:20 mix) according to literature precedent. Aliquots of the washed cell suspensions were added to a final concentration of 0.5 mg mL\(^{-1}\) dry weight biomass, by use of a syringe fitted with a needle, to anaerobic bottles sealed with butyl rubber stoppers containing uranyl(VI) acetate (5mM) in NaHCO3 buffer (30 mM, pH 7) creating the microcosm for the experiment. Lactate (10mM) was added to the microcosm as the electron donor. Samples were removed from the microcosm every hour, by use of a syringe fitted with a needle and filtered into a low temperature quartz EPR tube or placed onto a quartz slide and sealed with a cover slip for low temperature luminescence (flash freezing in liquid nitrogen at 77 k) and microscopy studies respectively. Steady state emission spectra were recorded on an Edinburgh Instrument FP920 Phosphorescence Lifetime Spectrometer equipped with a 5 watt microsecond pulsed xenon flash lamp and a red sensitive photomultiplier in peltier (air cooled) housing. The 1 photon-excitation FLIM-PLIM is a commercial system from Becker and Hickl with a 405 nm pulsed laser system. Fluorescence lifetime images were obtained using a modified confocal microscopy apparatus, constructed in the Central Laser Facility, which has a Nikon EC1 scanhead. Laser light was focused through a x60 water immersion objective with an NA of 1.2 on an inverted Nikon microscope (TE2000-U). Steady state spectra were recorded using an Ocean Optics USB2000+ spectrometer.
Results and Discussion

Uranyl(VI) luminescence is widely accepted to be a phosphorescent ligand-to-metal charge transfer process involving the axial oxygen molecules. This characteristic emission is typically centred around ca. 520 nm and up to six vibrational hot bands can be observed. Long lived lifetimes can also be observed due to the long lived excited state. The combination of this technique with microscopy allows fluorescence images to be obtained without the use of molecular dyes and plotting the changes in uranyl(VI) lifetime values using lifetime image mapping can provide further information on interactions at the cell surface.

S. oneidensis; JG 274 – wild type, JG 596 – lacking OM cytochromes, JG 1453 – IM and OM cytochromes, JG 1758 – lacking the ability to secrete extracellular flavins, were resuspended in 30 mM bicarbonate buffer supplemented with uranyl(VI) acetate (5 mM) and sodium lactate (10 mM) as an electron donor for the radionuclide reduction in a sealed serum bottle under a nitrogen atmosphere.

We previously reported that the emission of uranyl acetate added to cultures of Geobacter sulfurreducens and analysed in a frozen solution at 77 K is significantly more resolved than room temperature emission with mono-exponential kinetics suggesting the presence of a single emissive species. This was observed for all four strains of Shewanella oneidensis with uranyl lifetimes of 1040.60 ± 1.059 μs (JG 274 – wild type), 1024.56 ± 0.948 μs (JG1758 – lacking ability to secrete extracellular flavins), 974.52 μs ± 1.149 (JG 596 – lacking OM cytochromes) and 1000.70 μs ± 1.115 (JG 1453 – lacking IM and OM cytochromes) observed, although comparison with literature values for uranyl carbonate and hydroxide species did not allow identification of the emissive species in this system due to the complicated nature of the uranyl(VI) speciation.

The concentration of uranyl(VI) was monitored using luminescence spectroscopy over the course of a bioreduction experiment for each S. oneidensis bacterial strain. At hourly time points for the initial 8 hours and then again at 24 hours, an aliquot of the solution was removed from the reduction microcosm and frozen in liquid nitrogen where a fluorescence spectrum was obtained under standardised instrumental set up (see experimental for full details). A small amount of the supernatant, removed via a syringe filter to remove any biomass, was also submitted for liquid scintillation counting. The data reported are an average of three independent bioreduction experiments for each bacterial strain.

For the JG274 (wild type) strain and the JG1758 (lacking ability to secrete extracellular flavins) strain the fluorescence intensity of uranyl(VI) showed a general decrease and after 24 hours and 48 hours respectively solutions were non emissive, suggesting that the microbial reduction had proceeded to completion. This observation was supported by liquid scintillation counting which also showed a general decrease in soluble uranyl concentration (Figure 1).

Previous research observed during the bioreduction of uranyl(VI) acetate with Geobacter sulfurreducens highlighted a `saw-tooth pattern’ during monitoring of the reduction on concentration of uranyl(VI) by luminescence spectroscopy. The initial decrease in total emission intensity of the uranyl(VI) signal and subsequent sharp increase observed in hours 2-3 of the bioreduction process and further steady decrease in emission from hour 4 onwards, was attributed to the formation and rapid disproportionation of unstable metastable uranyl(V) (UO$_2^-$) intermediate which is non-emissive under the experimental conditions employed.

This same effect is observed during the reduction with Shewanella oneidensis, with a ‘saw-tooth’ pattern obtained reproducibly for JG274 which is attributed to the formation of an unstable UO$_2^{2+}$ intermediate which readily disproportionates to form the observed UO$_2^{3+}$ and UO$_{4}^{3+}$ species (Figure 1, Supporting Info 1). This suggests that the bioreduction process in wild type Shewanella oneidensis involves a one-electron reduction followed by disproportionation, as has previously been observed for other uranium reducing bacteria such as Geobacter sulfurreducens. The reduction of uranum by Shewanella oneidensis has not previously been studied in such detail and therefore this indication of a one-electron reduction followed by disproportionation is a novel discovery.

Figure 1: Plots of luminescence intensity (black) of uranyl(VI) normalised to time = 0 and liquid scintillation counting data (blue) for Shewanella oneidensis JG274 – wild type (above) and Shewanella oneidensis JG1758 – lacking ability to secrete extracellular flavins (below) over the course of a 24 hour reduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor.

It can also be seen in Figure 1 that the decrease in uranyl(VI) emission in the JG1758 strain seems to occur at a faster initial rate, with reaction rate values of 0.08 intensity hr$^{-1}$ for JG274 and 0.21 intensity hr$^{-1}$ for JG1758 (graphical analysis shown in SI figures 2 and 3). The presence of flavins may be affecting the results obtained as flavins are hypothesised to quench uranyl(VI) emission. This
effect is also observed during the reduction experiments (SI figure 4) when the luminescence intensity at time = 0 is compared to a uranyl(VI) acetate emission spectrum which has not been in contact with bacteria. The uranyl(VI) emission of the JG274 (wild type) decreases dramatically in the presence of the bacteria which are secreting flavins, whereas a the smaller decrease in emission intensity is observed for the JG1758 strain which is not secreting flavins and therefore not quenching the uranyl(VI) emission.

A titration carried out with oxidised flavin mononucleotide (FMN) shown in Figure 2 demonstrates the complete quenching of uranyl(VI) emission following the addition of 0.08 equivalents of FMN. Flavins are well known in biological process to be 2-electron reducing agents\(^ {17} \) but as both the flavin and uranyl(VI) were in oxidised forms it seems unlikely that reduction is occurring and the decrease in emission is more likely to be due to the quenching effect by energy transfer.

![Figure 2: Titration of oxidised flavin mononucleotide (FMN) in 0.025 mM aliquots steps from 0 mM to 0.4 mM to a uranyl(VI) acetate solution (5 mM) in a 30 mM bicarbonate buffer. Above, steady state emission spectrum of uranyl(VI) versus time at 77 K following 420 nm excitation and below, plot of uranyl(VI) emission intensity versus concentration of added FMN.](image)

When following uranyl(VI) concentration for the JG596 (lacking OM cytochromes) and JG1453 (lacking IM and OM cytochromes) strains (Figure 3) an initial decrease in uranyl(VI) emission is observed which could be due to sorption of the uranyl(VI) onto the bacterial cells before being released back into solution without reduction occurring. This, and the decrease in concentration shown by liquid scintillation data, suggests that there are other uranyl(VI) sorption sites on the surface of the bacteria, as there is still sorption occurring when the c-type cytochromes are absent. Over a 24 hour period there is little decrease in the emission intensity of the uranyl(VI) which suggests that the cytochromes on both the inner and outer membrane of the bacteria play an important role in the electron transport chain during the reduction process. This can be seen by the intensity of the uranyl(VI) emission at 24 hours, which indicates the presence of uranyl(VI) in high concentrations (0.65 a.u. and 0.82 a.u. respectively) compared to the reductions by JG274 and JG1758 where most of the uranyl(VI) has been reduced within 24 hours.

To investigate whether the cytochromes were capable of reducing uranyl(VI) directly, a titration was carried out with pure reduced cytochrome c from equine heart (Figure 4). Over the course of the titration, the intensity of the uranyl(VI) emission observed decreased, which was complemented by an increase a broad emission band at ca. 350 nm. This may be due to the presence of uranium(IV) species that has been formed by reduction of the uranyl(VI) by the cytochrome c, as broad emission bands for uranium(IV) has been reported in the literature at 365 nm.\(^ {28} \) Lifetime data was not available due to the short lived nature of the band at ca. 350 nm. Alternatively, the appearance of this shorter wavelength emission band may be due to the fluorescence from the haem centre of cytochrome c.

![Figure 3: Plots of luminescence intensity (black) of uranyl(VI) normalised to time = 0 and liquid scintillation counting data (blue) for Shewanella oneidensis JG596 – lacking OM cytochromes (above) and Shewanella oneidensis JG1453 – lacking IM and OM cytochromes (below) over the course of a 24 hour reduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor.](image)

An analogous process was then studied by fluorescence and phosphorescence confocal microscopy at sub-micron resolution to probe the distribution of uranyl within the microbial cultures. Fluorescence microscopy techniques such as Fluorescence Lifetime Image Microscopy (FLIM) and Phosphorescence Lifetime Image...
Microscopy (PLIM) can be used to spatially resolve the lifetimes of species present with sub-micron resolution. This enables in situ images to be taken during a bioreduction process enabling more information to be gained on the interactions occurring between the uranium and the cell surface. Both one- and two-photon techniques can be used allowing for a deeper investigation into the speciation of the uranium present. The experimental procedure was carried out as described previously with aliquots being placed on a glass slide and carried out at room temperature.

Figure 4: Steady-state luminescence spectra showing the decrease in uranyl(VI) emission and the increase in the emission band at ca. 350 nm with increased cytochrome c concentration during a titration of 0.1 mM reduced cytochrome-c into a uranyl(VI) acetate (1 mM) solution in a 30 mM bicarbonate buffer (above), and a zoomed in version to demonstrate the band at ca. 350 nm (below). Excitation wavelength 420 nm.

Little or no auto-fluorescence was observed from the bacteria upon excitation with a 405 nm laser in the on-photon confocal experiment, but upon the addition of uranium JG274 (wild type) and JG1758 (lacking ability to secrete extracellular flavins), which have both been shown to reduce U(VI) in work presented above, changeable lifetimes were apparent across the surface of the bacteria at the start of the bioreduction (time = 0). Images shown in figure 5 (with time = 2 data shown in supporting information for comparison), show lifetime ranges from 462 ps to 1287 ps and 725 ps to 1287 ps respectively, suggesting the uranyl(VI) emission is undergoing a competitive quenching process. The quenching of uranyl(VI) emission occurs when a non-radiative pathway for electron relaxation is present in the sample. This could occur due to changes in solvent speciation, changes in complexation or vibrational quenching due to the presence of O-H bonds. In this case, since vibrational quenching is likely to be time averaged, it is likely to be directly related to the interactions between the uranyl(VI) and a surface bound electron donating species on the bacterial cell, as there is no significant change in the solvent system or the presence of O-H bonds in the samples.

Intriguingly, the same variation in lifetimes were not observed in JG596 (lacking OM cytochromes) and JG1453 (lacking IM and OM cytochromes). These are the strains which do not appreciably reduce the UO$_2^{2+}$, indicating that the species responsible for the quenching of the uranyl emission are not present in these deletion mutants, which suggests it may be related to the absence of key c-type cytochromes in these mutants. The absence of cytochromes in the JG596 and JG1453 mutant strains also results in an inability to reduce the UO$_2^{2+}$ as seen in results presented earlier in this paper. This suggests that the cytochromes play an important role during the electron transfer pathway in the bioreduction of uranyl(VI), directly interacting with the uranyl(VI) on the surface of the bacteria causing the quenching of the emission observed in the samples where uranyl(VI) is successfully reduced.

Figure 5: One-photon fluorescence lifetime image microscopy map of lifetime data for S. oneidensis JG274 – wild type (left) and JG1758 – lacking ability to secrete extracellular flavins (right), at T=0 during a 24 hour reduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor.

This is supported by two-photon spectroscopy which can provide extra detail due to the increased resolution from the lower energies used in the process. Since the simultaneous absorption of two photons occurs in a small focal volume (down to a femtolitre), localised excitation results, which can help provide further
information on the interactions on the surface of the bacteria and increase the spatial resolution of the image.28,30

As can be seen in figure 7, the lifetimes across the surface of the JG274 (wild type) and JG1758 (lacking ability to excrete extracellular flavins) show a large variety compared to the JG596 (lacking OM cytochromes) strain. Analysis of the JG1453 (lacking IM and OM cytochromes) sample was not possible due to a weak signal. This could be due to the lack of uranyl(VI) sorbed onto the surface of the bacteria due to the lack of sorption to c-type cytochromes. This hypothesis is supported by the fact that the JG596 (lacking OM cytochromes) strain was not as emissive as the JG274 (wild type) strain. The most emissive sample was the JG1758 (lacking ability to secrete extracellular flavins) strain. Flavin cofactors, in this case, FMN are hypothesised to quench uranyl(VI) emission by resonant energy transfer (oxidised form) and via electron transfer processes in the reduced form and therefore when their secretion by the bacteria is hindered they no longer quench the emission. This quenching effect suggests there is a direct interaction between the FMN and the uranyl(VI) species which may impact on the bioreduction process as discussed previously.31

Conclusions

In summary, we have demonstrated the ability of Shewanella oneidensis MR-1 to reduce UO$_2^{2+}$ over a 24 hour period with the use of steady state and time resolved luminescence spectroscopy, one-photon fluorescence and phosphorescence confocal microscopy and lifetime image mapping. The ease at which these samples can be manipulated and analysed has revealed important mechanistic information regarding the bioreduction process and for the first time, a one electron reduction step is implied producing a metastable uranyl(V) intermediate that disproportionates to uranyl(VI) and uranium (IV). Experiments with three deletion mutant strains of Shewanella oneidensis have provided a much deeper insight into the role of the inner and outer membrane c-type cytochromes and extracellular FNM; JG274 (wild type), JG596 (lacking OM cytochromes), JG1453 (lacking IM and OM cytochromes) and JG1758 (lacking ability to secrete extracellular flavins). Steady state luminescence spectroscopy of the bioreduction process by these strains have shown that those lacking in cytochromes, JG596 (OM) and JG1453 (IM and OM), do not reduce the UO$_2^{2+}$ over the same time period as those which do, and that even a small difference in inner and outer membrane cytochromes affects the rate at which reductions occur. A combination of luminescence spectroscopy and X-ray based techniques currently used may be able to expand this knowledge further in future.

Both one and two photon fluorescence microscopy has suggested that the presence of the cytochromes on the bacterial surface has a significant quenching effect on the luminescent lifetime of any uranyl(VI) present, observed by the lack of variation in lifetime across the bacterial surface when the inner or outer cytochromes are not present. This suggests that the cytochromes are directly interacting with the uranyl(VI) on the surface of the bacteria, indicating that the reduction must be a direct process between the two species. Results also suggest that bound flavins may be disruptive to this process. This confirms that c-type cytochromes play an important role in the electron transport chain during the bioreduction of uranium, but that their interaction with uranyl(VI) may be more direct than previously considered.

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References

Electronic Supporting Information (ESI) for An investigation into the Role of c-type Cytochromes and Extracellular Flavins in the Bioreduction of Uranyl(VI) Acetate by *Shewanella oneidensis* using Fluorescence Microscopy.

D.L. Jones, a M.B. Andrews, a S.W. Botchway, b A. Ward, b J. R Lloyd, c L.S. Natrajan a

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**Figure S1:** Repeat bioreduction experiments of *Shewanella oneidensis* JG274 (wild type) over the course of a 24 hour reduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor, demonstrating the reproducibility of the ‘saw-tooth’ pattern.
Figure S2: Plot of the average *Shewanella oneidensis* JG274 uranyl(VI) luminescence intensity, taken over 3 repeats during the course of a 24 hour reduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor. Data analysed assuming first order reaction kinetics to obtain a reaction rate value. 

\[ y = -0.0843x + 11.938 \]

\[ R^2 = 0.9092 \]

Figure S3: Plot of the average *Shewanella oneidensis* JG1758 uranyl(VI) luminescence intensity, taken over 3 repeats during the course of a 24 hour reduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor. Data analysed assuming first order reaction kinetics to obtain a reaction rate value. 

\[ y = -0.2107x + 12.668 \]

\[ R^2 = 0.9187 \]
Figure S4: Averaged luminescence data for *Shewanella oneidensis* JG274 – wild type (black) and *Shewanella oneidensis* JG1758 – lacking ability to secrete extracellular flavins (blue) taken over 3 repeats during the course of a 24 hour reduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor. Data at time = -1 represents uranyl(VI) acetate emission before the addition of bacteria to the sample.

Figure S5: One-photon fluorescence lifetime image microscopy map of lifetime data for *S. oneidensis* JG274 – wild type (left) and JG1758 – lacking ability to secrete extracellular flavins (right), at T=2 during a 24 hour reduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor.
Figure S6: Histogram depicting the variation in lifetimes across the 1-photon FLIM image for *Shewanella oneidensis* JG274 (wild type) at T=2 during a 24 hour reduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor.

Figure S7: Histogram depicting the variation in lifetimes across the 1-photon FLIM image for *Shewanella oneidensis* JG1758 (lacking ability to secrete extracellular flavins) at T=2 during a 24 hour reduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor.
Figure S8: Histogram depicting the variation in lifetimes across the 1-photon FLIM image for *Shewanella oneidensis* JG596 (lacking OM cytochromes) at T=2 during a 24 hour reduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor.

Figure S9: Histogram depicting the variation in lifetimes across the 1-photon FLIM image for *Shewanella oneidensis* JG1453 (lacking IM and OM cytochromes) at T=2 during a 24 hour reduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor.

Intention to submit to Dalton Transactions.

More recent research into the bioreduction of uranium has included investigating the uranium(IV) products formed. For the technique to be useful for remediation studies, the uranium(IV) product must be less soluble that the original uranyl(VI) product to prevent further migration within the environment. Early work suggested that uraninite, a black insoluble mineral species, was the most commonly formed uranium(IV) product.

However, another uranium(IV) product has been reported which may be more soluble than uraninite. ‘Monomeric’ uranium(VI) has been reported in systems where higher quantities of phosphate is present, either during the growth of the cells or in the buffer used during the reduction.

This chapter investigates the uranium(IV) products formed during reduction by *Shewanella oneidensis* in either a bicarbonate or phosphate buffer. Uranium(IV) luminescence in aqueous systems is not well understood and therefore standard experiments to determine the applicability of this technique were undertaken. Fluorescence microscopy may help elucidate the form of the uranium(IV) product as uraninite is non-emissive whereas ‘monomeric’ uranium(IV) may be emissive and therefore identifiable under certain conditions.

All the experimental data was collected and analysed by the author, including bacteria growth, uranium luminescence experiments and fluorescence microscopy work carried out at the Rutherford Appleton Laboratories (RAL) in Oxford. This chapter was also written by the author. M.B. Andrews provided support during the development of experiments and during the analysis of data. S.W. Botchway and A. Ward provided help during the experimental set-up of the fluorescence microscopy data used at RAL. J.R. Lloyd (microbiology) and L.S. Natrajan (spectroscopy) were the academic supervisors for this piece of work.
An Investigation into the Uranium(IV) Product Formed after Bioreduction of Uranyl(VI) Acetate by *Shewanella Oneidensis* using Optical Spectroscopy and Microscopy.

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The emission of uranium(IV) in water and phosphate buffered saline (PBS) have been studied with an aim to utilise a range of optical spectroscopic techniques to study the formation of the uranium(IV) product in the bioreduction of uranyl(VI) acetate with lactate as the electron donor, by the Gram-negative metal reducing bacteria, *Shewanella oneidensis*. Initial studies included luminescence spectroscopy of uranium(IV) tetrachloride in water and PBS using a range of excitation wavelengths; 245, 303, 325 and 420 nm. Broad emission observed at ca. 350 nm was assigned as a charge transfer type transition from the 6d1 5f2 excited state to the lower 5f2 states; F1, G1, D2, P1, F2, F3 and the H4 and H6, ground states. Studies across a wide pH range also demonstrated the applicability of this technique at biologically relevant conditions. Bioreduction experiments with *S. oneidensis* conducted in a phosphate buffer (PBS) have shown the ability to measure the emission of both uranyl(VI) and uranium(IV) in solution using both 420 and 245 nm excitation and suggested a 2-electron reduction process may be occurring due to an increase in U(IV) emission during the early stages of the experiment. Fluorescence microscopy showed the existence of a homogeneous uranyl(VI) phosphate precipitate with bacteria on the surface. A direct interaction between the bacteria and the surface of the precipitate is observed suggesting that for uranyl(VI) to be reduced by bacteria it does not necessarily need to be in solution. These techniques demonstrate the ability to investigate the uranium(IV) product of bioreduction during in situ experiments which could contribute to an enhanced knowledge of the bioreduction system for use in the safety case for deep geological disposal.

Introduction

Over the course of its 60 + year lifetime, the nuclear industry has amassed a significant quantity of radioactive nuclear waste, which, in the case of the United Kingdom is currently being stored at the Sellafield Site in Cumbria. 1 The majority of this waste is uranium, with the minor actinides and other fission products such as technetium adding to the principal radioactivity and heat loading of the spent nuclear fuel. 2 The need for both short to mid-term storage facilities and long-term disposal facilities, namely the world-wide proposed deep geological disposal facilities (GDF) to deal with this legacy waste are nowadays generally undisputed. However the safety cases involved are extremely complicated and implementation relies on a full understanding of the interactions of the radionuclides with the surrounding geosphere and biosphere. 3

The GDF proposal relies on a multi-barrier system to prevent the release of radionuclides into the environment over time. These include vitrification of the waste into a stable form, containment in a steel canister and a cementitious backfill. 4 However over the course of several millennia (the time it would take for radiation levels to reduce to non-hazardous levels to be considered non-toxic to life), the safety case has to take into account the fact that the radionuclides may well breach the repositories’ multi-barrier system, entering the local environment where transport away from the repository can occur. One of the final barriers in the UK proposal is the bio-barrier, formed from indigenous bacterial populations found in the sub surface. 5 Certain bacteria have been known to reduce toxic heavy metals, generally from a soluble mobile higher oxidation state species into a less soluble and therefore immobile lower oxidation state species 6 which would be of great utility in a GDF system. In the case of uranium, gram-negative metal reducing bacteria such as *Geobacter sulfurreducens* and *Shewanella oneidensis*, 7, 8, 9 have been shown to effectively reduce uranium from the water soluble uranyl(VI) ([UO\(_2\)]\(^{2+}\)) ion to a much less soluble uranium(IV) species, principally the insoluble mineral uraninite, UO\(_2\). 10 Although molecular uranium(IV) inorganic species (phosphates, carbonates, termed ‘nonmoneric uranium(IV)’) can also be formed via bioreduction with *Shewanella oneidensis* depending on the local concentrations of minerals in the vicinity of the uranium. 11
Initial research into the uranium(IV) species formed during the bioreduction process with *Shewanella oneidensis* found it to be a black mineral, uraninite.\(^{11,12}\) Further studies have found that uraninite formation typically occurs on or within the cell membrane, and can form nano-meter sized particulates.\(^{13,14}\) The formation of uraninite is important within the bioreduction as it is a relatively insoluble species and therefore may be immobile and less susceptible to re-oxidation within the environment which is a key feature in the bio-barrier safety case of the GDF.

However, recent studies suggest that a less insoluble form of uranium(IV) can be formed during the bioreduction.\(^{15}\) The formation of so called ‘monomeric’ uranium(IV) is emerging in both laboratory experiments and in natural *in situ* field experiments.\(^{16}\) The geochemical conditions have been found to control the uranium(IV) species produced,\(^{17}\) with the presence of phosphates and carbonates affecting speciation with some studies suggesting that ‘monomeric’ uranium(IV) is primarily associated with phosphates and carbonates on bacterial cell walls.\(^{18}\)

Studies have found that the media used to grow the bacteria can also have an effect with simple media such as Luria-Bertani (LB) media favouring the formation of uraninite with a more complex media such as Widdel Low Phosphate (WLP) media favoring the formation of ‘monomeric’ uranium(IV).\(^{19}\) This is also true for phosphate free media which favours the formation of uraninite while phosphate amended media favours the formation of ‘monomeric’ uranium(IV) such as [CaU(PO\(_4\))\(_2\)]\(_{10}\)U\(_2\)O\(_{23}\) and U\(_2\)PO\(_4\)(P\(_2\)O\(_7\)).\(^{17}\) Thus suggests the role of phosphate is crucial in determining which uranium(IV) product is formed.

The uranium(IV) products resulting from bioreduction of uranyl(IV) with bacterial populations are typically investigated using X-Ray based techniques as uraninite contains a U-U bond length of 3.85 Å whereas this is not be present in ‘monomeric’ uranium(IV).\(^{11}\) These techniques can be time consuming and involve complicated sample preparation. It is also difficult to follow the reduction process *in situ* with these techniques. On the other hand, luminescence spectroscopy, which is a very sensitive spectroscopic technique, has the potential to distinguish between the different oxidation states of uranium (here uranyl(IV) and uranium(IV)) by exploiting the different absorption, excitation and emission profiles at much lower concentrations (sub millimolar) and importantly, can provide insight to the speciation occurring using kinetic lifetime data.\(^{20}\)

The emission from uranyl(IV) is generally accepted as a ligand-to-metal charge transfer (LMCT) from the uranyl oxygen 2p orbitals to the empty uranium 5f orbitals, with the electronic transition typically centred around ca. 520 nm and can contain up to 6 vibrationally resolved hot bands, with long-lived lifetimes of up to 180 μs.\(^{21}\) Uranium(IV) emission typically arises due to intra f-f transitions, resulting in line like emission spectra corresponding to various relaxation pathways within the 5f electronic manifold with shorter lifetimes of <0.02 μs.\(^{21}\) Kirishima *et al* observed uranium(IV) emission in perchloric acid at pH 1, using an excitation wavelength of 245 nm, resulting in 10 peaks at 525, 409, 394, 345, 338, 335, 320, 318, 291 and 289 nm.\(^{22}\) These transitions are assigned as relaxation from the \(^{1}S_{0}\) \(^{5}F_{2}\) excited state to various ground states but in non-aqueous solutions where the surrounding ligands are no longer H\(_2\)O, the \(^{1}F_{2}\) excited state is lower in energy which results in three charge transfer broad emission bands at roughly 365, 410 and 490 nm.\(^{23}\)

Uranium phosphates are insoluble in solution and therefore have only been partially investigated using luminescence techniques.\(^{24}\) Uranyl(VI) phosphates, U\(_3\)O\(_7\)(H\(_2\)PO\(_4\))\(_2\), U\(_3\)O\(_7\)HPO\(_4\), UO\(_2\)PO\(_4\) and UO\(_2\)PO\(_4\) in the 1 to 5 pH range were studied by Cortina *et al.*, who characterized the emission spectra and lifetimes for a range of uranyl(VI) phosphate complexes. The resulting emission spectra contained peaks at 500, 520 and 540 nm with lifetimes of roughly 30 μs.\(^{25}\) Uranium(IV) phosphates have been studied at pH 1 by Lehman *et al* with a broad emission feature spanning 270–460 nm, superimposed upon which were two sharper bands at approximately 320 and 410 nm. However, the origins of the emission bands were not defined. Although the U(IV) phosphate product was non-emissive in this study, addition of phosphate to U(IV) had a noticeable quenching effect on the emission of the initial U(IV) present in solution.\(^{26}\) Uraninite has also been investigated using fluorescence spectroscopy and was found to be non-emissive following 266, 355, 409 or 532 nm excitation.\(^{27}\) These examples suggest that luminescence spectroscopy has great potential in elucidating the role of ‘monomeric’ uranium(IV) in the bioreduction of uranium.

In this paper, we use a variety of optical spectroscopic techniques in an attempt to validate both the presence of uranium(IV) molecular species which are anticipated to be partially soluble as they form and elucidate the role of ‘monomeric’ uranium(IV) within the bioreduction of uranium by *Shewanella oneidensis* JG274 – an unaltered wild type strain of the bacteria.\(^{28}\) A combination of luminescence spectroscopy and fluorescence microscopy are used to identify the formation of soluble uranium(IV) species within the microcosm and elucidate its role within the bio-reduction.

**Results and Discussion**

To begin with, samples prepared anaerobically, containing uranium(IV) were studied to investigate the ability to detect this oxidation state in water at neutral pH and at low temperature (77 K). Low temperature studies were conducted to maintain consistency between results as the bioreduction luminescence experiments were carried out at low temperature. The emission from optically dilute samples of 5mM UCl\(_4\) in degassed water prepared under inert atmospheric conditions were studied at several excitation wavelengths (245, 303, 325 and 420 nm). Excitation wavelengths of 245, 303 and 325 nm result in a broad emission band at ca. 350 nm in agreement with the work by Lehman *et al.*\(^{29}\) but dissimilar to the more lanthanide-like multiple sharper emission bands observed by Kirishima *et al.* in 1 M perchloric acid solution.\(^{22}\) This broader transition instead more resembles a charge transfer type transition and has previously been assigned electronic transitions originating from the from the U(IV) 6d\(^{5}\)F\(^{1}\) \(^{5}\)F\(^{2}\) excited state to the 5f\(^{2}\) lower energy spin orbit coupled states \(^{2}F_{7/2}\) \(^{5}G_{7/2}\), \(^{2}D_{5/2}\), \(^{2}P_{1/2}\) \(^{2}F_{5/2}\) \(^{2}P_{3/2}\) \(^{2}F_{3/2}\) \(^{2}P_{1/2}\) and the \(^{3}H_{4}\) and \(^{3}H_{6}\) ground states. Excitation at 420 nm results in a large broad band at 450 nm that may incorporate these transitions but is...
overshadowed by a larger broader signal. However, at 77K it is noteworthy that no vibrationally resolved emission attributable to uranyl(VI) is observed around 520 nm.

![Graph showing luminescence intensity over wavelength and time](image)

Figure 1: Low temperature (77 K) emission spectra for uranium(IV) tetrachloride (5mM) in water at various excitation wavelengths; 245, 303, 325 and 420 nm. * indicates scatter peaks from the excitation source (blue trace) and from the aqueous buffer (pink, green and black traces).

Given that intra-f-f emissive transitions have been observed at pH 1 in perchloric acid solution, the optical profiles of UCl₄ in water over the pH range 1-11 was investigated using an excitation wavelength of 245 nm. This excitation wavelength was chosen to best avoid exciting any potential uranyl(VI) present that may occur by the ingress of air in the samples. The emission profiles at pH 1, 3, 5, 7, 9 and 11 are all essentially the same and are similar to that recorded in PBS buffer. Interestingly however, the emission intensity is greatest at pH 1. These data clearly show that by exciting at 245 nm, uranium(IV) emission in solution is detectable- even at neutral pH which is important for biological samples and the emission profile is typical at all pH values and excitation wavelengths studied.

![Graph showing luminescence intensity over wavelength](image)

Figure 2: Low temperature (77 K) emission spectra, excited at 245 nm, of uranium(IV) tetrachloride (5mM) in water at various initial solution pH values, 1, 3, 5, 7, 9 and 11.

To visualize the uranium(IV) in solution a standard bioreduction experiment was conducted with *Shewanella oneidensis* JG274 (wild type) and low temperature measurements were taken, exciting at 420 nm to probe the uranyl(VI) emission and at 245nm to probe the uranium(IV) emission in solution. Although the reduction does not go to completion during the 24 time period that the experiment was conducted over, the reduction in uranyl(VI) concentration does decrease over time as can be seen in Figure 3. Unfortunately, the uranium(IV) signal over the course of the experiment was difficult to visualize as the peaks observed at 350 nm, which may have been due to U(IV) were quite weak in comparison to the uranyl(VI) species due to the much larger relative quantum yield of uranyl(VI) emission at 77K.

![Graph showing luminescence intensity over time](image)

Figure 3: Low temperature emission, excited at 420 nm, for *Shewanella oneidensis* over the course of a 24 hour reduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor in a bicarbonate buffer (30 mM).

By plotting the intensity of the peak maxima, at 503 nm over time, it becomes simpler to observe the changes in uranyl(VI) emission during the bioreduction experiment (Figure 4). The uranyl(VI) luminescence intensity follows the ‘saw-tooth pattern’ that is expected and previously observed indicating a one-electron reduction to unstable uranyl(VI) which then disproportionates to uranyl(VI) and uranium(IV), this has previously been reported for both *Geobacter sulfurreducens* and *Shewanella oneidensis*.\(^{20,21}\) In order to visualise the changes in uranium(IV) emission over the course of an experiment, the quantum yield of the emission must be increased, enabling stronger peaks at ca. 350 nm to be observed.

![Graph showing luminescence intensity over time](image)

Figure 4: Normalised emission for uranyl(VI) excited at 420 nm over the course of a 24 hour reduction with *Shewanella oneidensis*, uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor.

The presence of phosphate is known to enhance the total emission quantum yield of uranyl(VI) ions, and it follows that phosphate may well play a similar role in U(IV) species by aiding in vibrational isolation of U(IV) and hindering competitive non-radiative decay processes.\(^{22}\) Therefore, initial experiments were carried out with
UCl₄ (5 mM) in phosphate buffer (PBS) at pH 7.4 with the aim of being able to detect the uranium(IV) luminescence more clearly, either with bulk sampling or fluorescence microscopy. The presence of phosphate has also been reported in the literature to favour the formation of ‘monomeric’ uranium(IV) over the non-emissive mineral uraninite. Initial samples were run with UCl₄ (5 mM) in PBS and shown in Figure 5 below. Excitation wavelengths of 245 and 325 nm result in an emission band at ca. 350 nm and excitation at 303 and 325 nm also resulted in an emission band at ca. 420 nm. Excitation at 420 nm again resulted in a broad emission 450 nm. These samples proved to be more difficult due to the lower solubility of uranium phosphates, but measurements were still obtained in solution, at low temperature, and therefore a reduction experiment was carried out.

Figure 5: Low temperature (77 K) emission spectra, excited at 245 nm, of uranium(IV) tetrachloride (5mM) in phosphate buffer (10mM) at various initial solution pH values. * indicates scatter peaks from the excitation source (blue, pink and green trace at 350, 370 and 400 nm) and from the aqueous buffer (pink and green traces at 525 and 575 nm).

The early stages of the bioreduction of uranyl(VI) by Shewanella oneidensis in 10 mM PBS at pH 7.4 was monitored over 6 hours (Figure 6) and clearly shows the ability to monitor both uranyl(VI) and uranium(IV) luminescence simultaneously by excitation at 245 nm. This and the precipitation of uranium phosphates in the samples result in artifacts in some of the spectra obtained.

Figure 6: Low temperature (77 K) emission, excited at 245 nm, for Shewanella oneidensis over the course of a 24 hour reduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor in phosphate buffer (10 mM).

When plotting the change in emission intensity of uranyl(VI) and uranium(IV) during the first four hours of the reduction, where most of the changes in oxidation state occurs, it can be seen that after 1 hour there is a decrease in the uranyl(VI) emission which is complemented by an increase in the uranium(IV) emission. This suggests a direct reduction from the uranyl(VI) to a soluble form of uranium(IV), potentially ‘monomeric’ uranium(IV), promoted by the presence of phosphates in the buffer. An increase in the uranyl(VI) concentration is then observed which may be reoxidation of the soluble uranium(IV) as the concentration of uranium(IV) in solution decreases slightly. There may also be disproportionation of some uranyl(V) intermediate which has been previously observed for this strain of Shewanella oneidensis which may lead to slight discrepancies in the luminescence intensities. This, in combination with the onset of precipitation of nanoparticulate uraninite throughout the bioreduction process may account for the non-linear trend in the intensity of the emission signal in the uranium(IV) region (300-500 nm). Nonetheless, it is evident that there is an inverse correlation between the relative intensities of U(IV) and uranyl(VI) emission features (Figure 7).

Luminescence microscopy has further been used to gain insight to the processes occurring during the bioreduction in the presence of phosphate. Unfortunately, the experimental set-up did not enable excitation between 245-330 nm which is the optimal spectral excitation window for uranium(IV) emission, so the emission from uranyl(VI) alone was investigating following direct excitation at 405 nm. One photon fluorescence lifetime image microscopy (FLIM) and phosphorescence lifetime image microscopy (PLIM) of samples prepared on glass slides at room temperature at the very beginning of the bioreduction process showed the existence of a mass of precipitate containing long lived uranyl(VI) species within the samples, with phosphorescence lifetimes of up to 7 μs; no shorter lived fluorescence was detected. Figure 8 depicts the PLIM data for the sample recorded 2 hours after the bioreduction reaction was initiated and shows a uniform distribution of lifetimes across the surface of the precipitate with no discernible features, emission signal observed on the individual bacteria or bacterial colonies within the sample.
By contrast to the one photon FLIM and PLIM confocal microscopy studies, two-photon microscopy (using an excitation wavelength of 810 nm), shown in Figure 9 below, resulted in a much clearer image of the precipitated uranyl(VI) species, and in the FLIM window, also highlights the location of the bacteria within the sample. They can clearly been seen on or very near to the uranyl(VI) precipitate, suggesting there is uranyl(VI) sorbing onto the surface of the bacteria. However no fluorescence was observed for those bacteria in the FLIM temporal window which are not in close proximity to the precipitate suggesting that in the solid phase a direct interaction is required between the bacteria surface and uranyl(VI).

Conclusions

In summary, we have demonstrated the ability to measure uranium(IV) emission in water and phosphate buffer saline (PBS) buffer using range of UV excitation wavelengths. We have also demonstrated the ability to measure uranium(IV) emission at neutral, biologically relevant pH’s, reducing the need for low pH studies. Bio-reduction experiments with Shewanella oneidensis, uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor in a phosphate (10 mM) buffer has shown the ability to monitor the decrease of the uranyl(VI) steady state emission intensity alongside the increased emission intensity of soluble uranium(IV) using 245 nm excitation. These experiments have highlighted the increase in soluble uranium(IV) concentration early within the reduction process suggesting a 2-electron reduction step may be occurring. This may be occurring in tandem with the previously reported\(^{30,31}\) one-electron reduction, which is then followed by disproportionation since this is also evident in these samples through the ‘saw-tooth’ pattern observed in the uranyl(VI) emission.

One- and two-photon fluorescence microscopy show the existence of a homogenous uranyl(VI) precipitate, which exhibits long-lived emission with with lifetimes of up to 9 μs recorded, which is likely to be uranyl(VI) phosphate. Two-photon spectroscopy resulted in more spatially resolved images showing the bacteria within this precipitate with much shorter lifetimes of 1 ns on the surface. This suggests a direct interaction between the bacteria and the precipitated uranyl(VI) with species on the surface of the bacteria significantly quenching the luminescence lifetimes, indicating that for uranyl(VI) to be reduced by bacteria it does not necessarily need to be in solution, although the rates of the reduction are dramatically reduced.
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References

7.0 Conclusions & Future Work

Due to the radioactive contamination of land by radionuclides, in particular uranium, arising from 60+ years of nuclear fission activities, remediation strategies are required to clean up many legacy sites across the world. There are many potential remediation strategies including chemical leaching and digging up the contaminated land for disposal elsewhere. One of the most environmentally friendly methods being considered is to use anaerobic indigenous bacteria present in subsurface soils to reduce mobile uranyl(VI) (U\(^{IV}\))\(_2\)\(^{2+}\) species to a more immobile uranium(IV) mineral phase such as uraninite. This process is known as bioreduction. Another use for bioreduction is in the world wide proposed deep geological disposal facilities where a multi-barrier system incorporating a final natural ‘bio-barrier’ that separates the stored nuclear waste containers from the natural environment host rock makes up the safety case involved.

The bioreduction process has been extensively studied in the literature and yet due to the complicated nature of the process and the genetic/bio diversity in bacteria that can be involved, the process is far from being fully understood. The generally accepted mechanism involves the oxidation of an organic electron donor such as acetate or lactate, followed by transfer of one or more electrons within the bacteria via the bacterial electron transport chain. This ultimately results in the reduction of uranyl(VI) to uranium(IV). However, the role of the putative kinetically and thermodynamically unstable uranyl(V) intermediate which has been proposed to exist in certain systems which then disproportionates to form uranyl(VI) and uranium(IV) warrants further investigation and validation.

There were two main aims to this research project; the first to use a variety of optical spectroscopic techniques to investigate the bioreduction of uranium by *Geobacter sulfurreducens* and *Shewanella oneidensis* to further the understanding of the bioreduction process, including interactions at the cell surface of the bacteria. The second aim was to demonstrate the applicability of luminescence spectroscopy and fluorescence microscopy to these systems, using the inherent uranyl(VI) emission to investigate the process, avoiding the use of fluorescent dyes which may complicate the system.

Initial results with *G. sulfurreducens* demonstrated that low temperature luminescence spectroscopy was able to detect uranyl(VI) reduction with a ‘saw-tooth’ pattern around hour 3 or 4 observed in the intensity of the uranyl(VI) emission (i.e. an initial decrease
in emission intensity followed by a sharp increase then overall decrease) indicating the formation of a uranyl(V) intermediate which subsequently undergoes disproportionation to uranyl(VI) and uranium(IV).

One-photon fluorescence microscopy was used, for the first time, to map the lifetimes of uranyl(VI) across the surface of the bacteria. This uncovered two previously unconsidered steps in the process, the sorption of uranyl(VI) onto the surface of the bacteria and the formation of large (ca. 20 µm diameter) extracellular heterogenous features containing uranyl(VI) and extracellular polymeric substances, most probably phosphate and acetate. Two-photon spectroscopy uncovered a vast variation in uranyl(VI) luminescence lifetimes across the surface of the bacteria which may be due to quenching effects caused by surface bound electron rich moieties such as solvent free multi-hemes of c-type cytochromes.

To progress this further, three deletion mutants of *S. oneidensis* were investigated. Two of the strains, JG596 and JG1453 lacking differing amounts of c-type cytochromes enabled direct comparison of the bioreduction rate in both, the presence and absence of inner and/or outer membrane c-type cytochromes. Results showed that bacteria where the cytochromes were absent, were not able to reduce uranyl(VI) as efficiently as those containing cytochromes, implying that the c-type cytochromes play a crucial role in the electron transport chain within the bacteria. Fluorescence microscopy indicated there was a variation in uranyl(VI) lifetimes across the surface of the *S. oneidensis* strains that were able to reduce uranyl(VI), as had previously been observed in *G. sulfurreducens* samples, but that those lacking in cytochromes had a consistent lifetime across the cell surface. This further supports the hypothesis that there is a direct interaction between the uranyl(VI) metal ion and the surface of the bacteria and in fact suggests that it may be the outer membrane cytochromes that the uranyl(VI) cations are interacting with.

Combined together, these results suggest that the reduction of uranyl(VI) by Gram-negative bacteria such as *G. sulfurreducens* and *S. oneidensis* begins with sorption of the soluble uranyl(VI) onto the surface of the bacteria where they are directly interacting with surface bound species, namely the outer membrane cytochromes and cellular polymeric substances. Absence of the cytochromes hinders the reduction process and therefore it is hypothesised that these are directly involved in the reduction pathway as key members of the electron transport chain. These cytochromes then transfer the electron to the sorbed uranyl(VI) causing a one-electron reduction to the
unstable uranyl(V) intermediate which subsequently undergoes disproportionation to uranyl(VI) and uranium(IV), whereby all the uranyl(VI) is then consumed and converted to the final uranium(IV) product.

The uranium(IV) product that is usually formed, especially in laboratory experiments is a black mineral, uraninite, which can be observed as the reduction proceeds. However, recent literature has suggested that there may be a more soluble form of uranium(IV); a molecular inorganic compound known as ‘monomeric’ uranium(IV). Optical spectroscopy and microscopy were used to investigate the formation of the uranium(IV) product in an attempt to identify any ‘monomeric’ uranium(IV) produced. Reduction by *S. oneidensis* in a phosphate buffer system at pH 7 (phosphate buffered saline, PBS) resulted in an increase in soluble uranium(IV) detectable by luminescence spectroscopy. Fluorescence microscopy was not able to detect soluble uranium(IV) due to restrictions in excitation wavelength within the experimental set up, but was able to image uranyl(VI) phosphate precipitates with bacteria directly in contact with the solid also displaying uranyl(VI) emission indicating sorption of uranyl(VI) into the surface of the cells.

The work presented here also demonstrates the applicability of optical spectroscopy and microscopy for studying the bioreduction of uranium by different bacterial species for the first time. Luminescence spectroscopy enables *in situ* analysis of both uranyl(VI) and soluble uranium(IV) in the systems allowing a deeper understanding of the molecular processes occurring. More frequent sampling can be used to uncover previously unconsidered steps in the process which can aid the future understanding of the bioreduction of uranium. One-photon fluorescence microscopy, including fluorescence lifetime image mapping (FLIM) and phosphorescence lifetime image mapping (PLIM), utilising the inherent luminescence properties of uranyl(VI) allow for a sub micrometer resolution on bacterial cells without the use of additional dyes which may affect the interactions between uranyl(VI) and the bacteria. In addition, two-photon microscopy can significantly enhance spatial resolution (sub-micron) which can uncover further details on the surface of the bacteria which may have previously been overlooked.

These techniques, however, do have limitations. As the technique is still in its infancy, the equipment required, especially for the fluorescence microscopy is specialised and currently only available in certain research facilities. The identification of uranium(IV)
by luminescence spectroscopy is also in its infancy and therefore accurate literature data for comparison and spectroscopic techniques are still not widely available making any research into this area difficult. A combination of these optical spectroscopic techniques with the techniques currently employed in this field such as EXAFS could provide a broad range of complementary analysis techniques which would expand the current knowledge in this field.

Future work in this project would include the investigation of other indigenous bacteria, including Gram-positive bacteria, and moving towards more environmentally relevant samples to help establish the usefulness of optical spectroscopy further. Samples obtained from a contaminated site, such as the Sellafield site in the UK would be interesting to investigate as they would contain other contaminants which may affect the inherent luminescence of uranyl(VI). A mixture of different bacteria would also be more relevant to the natural and engineered environment and the use of fluorescence microscopy in combination with lifetime mapping could be extremely beneficial in identifying different (molecular) interactions between various bacteria and uranyl(VI) as well as between each other. The in situ nature of the technique could provide more insight to the processes occurring in the real environment, which could greatly enhance our understanding of biogeochemical processes and in turn advance and progress the safety case for geological disposal.

8.0 Experimental

_Uranyl Acetate_

For all experiments conducted on the uranium(VI) oxidation state, uranyl acetate was used. A 25 mM stock solution was prepared by dissolving $\text{UO}_2(\text{CH}_3\text{COO})_2\cdot\text{H}_2\text{O}$ ($\sim 0.5$ g) in deionized water (50 mL). Samples were removed from the stock solution for subsequent experiments.

_Uranium Tetrachloride_

For standard experiments conducted on the uranium(IV) oxidation state, uranium tetrachloride was used, all manipulations were carried out in an oxygen-free glove box. A 5 mM stock solution was prepared by dissolving $\text{UCl}_4$ ($\sim 0.5$ g) in deionized water (250 mL) or PBS buffer (250 mL, pH 7). Samples were removed from the stock
solutions for subsequent experiments. For pH measurements, a small volume of the stock was added to pre-calibrated pH solutions.

*Geobacter Sulfurreducens*

Cells were grown in NBAF growth medium (80 mL), with inoculation percentages of 5, 10 or 20% (5 and 10 % were to maintain a living culture, 20 % for harvesting for experiments). Samples were grown for 24 hours in a 30 ºC incubator before either being harvested or stored for later use in a 20 ºC incubator for a maximum of 2 weeks.

All manipulations of cells were carried out under an atmosphere of N₂. Late-log-phase cultures were harvested by centrifugation and washed twice in NaHCO₃ buffer (30 mM, pH 7, degassed with N₂-CO₂ 80:20 mix). Aliquots of the washed cell suspensions were added to a final optical density of 0.3, by use of a syringe fitted with a needle, to anaerobic bottles sealed with butyl rubber stoppers containing uranyl(VI) acetate (5mM) in NaHCO₃ buffer (30 mM, pH 7). No electron donor was added as the acetate from the uranyl acetate acts as the electron donor in this case.

Samples were removed from the cultures every hour, by use of a syringe fitted with a needle and filtered into a young’s tap cuvette, a low temperature EPR tube or placed onto a slide and sealed with a cover slip, for room temperature luminescence, low temperature luminescence (samples run at 77 K, by flash freezing with liquid nitrogen) and microscopy studies respectively.

For ‘dead cell’ experiments, the harvested bacteria were autoclaved at 126 ºC and a pressure 1 bar before being used in the same experimental conditions.

*Shewanella Oneidensis*

Cells were removed from frozen stock and grown on agar plates for 24 hours in a 30 ºC incubator. The agar plates were subsequently stored in a refrigerator for a maximum of 1 week. Small colonies were removed from the agar plate with an inoculation loop and transferred to an aerobic sample of LB broth (100 mL).

The inoculated LB broth was placed in a shaking incubator at 30 ºC, 60 rpm, for 24 hours before cells were harvested in an identical method to *G. sulfurreducens*, with all cell manipulations from this step onwards being carried out under an atmosphere of N₂. 10 mM lactate solution as the electron donor was added for all *S. oneidensis* experiments.
Some experiments with JG 274 were washed and carried out in a PBS buffer (10 mM, pH 7), all other experimental conditions remain the same. Samples were removed from the cultures every hour, in the same method as for *G. sulfurreducens*.

*Alkaliphilus Oremlandii*

Cells were grown in DMZ growth medium (250 mL), with an inoculation volume of 10 mL. Samples were grown for 3 days in a 30 °C incubator before either being harvested or stored for later use in a 20 °C incubator for a maximum of 3 weeks. All cell manipulations were carried out under an atmosphere of N₂. Cultures were harvested by centrifugations and washed twice in NaHCO₃ buffer (30 mM, pH 8.2, degassed with N₂·CO₂ 80:20 mix). Aliquots of the washed cell suspensions were added to a final optical density of 0.3, by use of a syringe fitted with a needle, to anaerobic bottles sealed with butyl rubber stoppers containing uranyl(VI) acetate (5mM) in NaHCO₃ buffer (30 mM, pH 8.2). 10 mM lactate solution as the electron donor was added to the cultures for all *A. Oremlandii* experiments. Samples were removed from the culture every hour, in the same method as for *G. sulfurreducens*.

*Escherichia Coli*

Cells were removed from frozen stock and grown in a small volume (1 mL) of LB broth solution in a 30 °C incubator for 2 hours. This inoculum was then transferred to an aerobic sample of LB broth (100 mL) and grown for a further 24 hours in a shaking incubator at 30 °C and 60 rpm. Cells were harvested in an identical method to *G. sulfurreducens*, with all cell manipulations from this step onwards being carried out under an atmosphere of N₂. 10 mM lactate solution as the electron donor was added for all *E. Coli* experiments. Samples were removed from the culture every hour, in the same method as for *G. sulfurreducens*.

*Flavin MonoNucleotide (FMN) Titration*

A 50 mM stock solution of FMN, in the reduced form, was made (22 mg, 1 mL). This was then added to an anaerobic serum bottle containing uranyl acetate (5 mM) in NaHCO₃ buffer (30 mM, pH 7). Low temperature emission spectra and lifetime data were taken upon the addition of FMN in 25 μM increments from 0 μM to 400 μM.

*Cytochrome C Titration*
A 1 mM stock solution of cytochrome C from equine heart, in the reduced form, was made (30 mg, 2 mL). This was then added to an anaerobic serum bottle containing uranyl acetate (1 mM) in NaHCO$_3$ buffer (30 mM, pH 7). Low temperature emission spectra and lifetime data were taken upon the addition of Cyt C in 0.1 mM increments from 0 mM to 0.7 mM.

Instrumental
Optical density was calculated using a UV spectrometer, Jenway model 6715 UV/Vis Spectrophotometer.

Steady state emission spectra were recorded on an Edinburgh Instrument FP920 Phosphorescence Lifetime Spectrometer equipped with a 5 watt microsecond pulsed xenon flash lamp and a red sensitive photomultiplier in peltier (air cooled) housing, (Hamamatsu R928P). Lifetime data were recorded following 245, 290, 303, 325 or 420 nm excitation with a 5 Watt xenon flash lamp (Edinburgh Instruments), using multichannel scaling. Lifetimes were obtained by tail fit on the data obtained, and quality of fit judged by minimization of reduced chi-squared and residuals squared. Settings varied across different experiments based on the intensity of the observed emission but were kept constant during individual experiments or repeat experiments. All spectra were run in triplicate.

For analysis of the samples using liquid scintillation counting, a 100 μl sample was mixed with scintillant (Scintisafe 3, Fisher Scientific) and 1 mL HCl prior to counting on a LSC (Quantulus, PerkinElmer) instrument. All samples were run in triplicate.

The uraninite powder was analysed via powder X-Ray Diffraction (pXRD; Bruker D8 Advance using Cu-Kα radiation and EVA 14 analytical software).

The 1 photon-excitation FLIM-PLIM is a commercial system from Becker and Hickl with a 405 nm pulsed laser system with 10 minute collection times. Two-photon time-resolved microscopy and imaging of Geobacter Sulfurreducens were performed by using a mode-locked Ti-sapphire laser (Mira, F900D, Coherent UK) operating at 810 nm with pulse length ~200 fs, 76MHz repetition rate, pumped by a Vanadate V18 green continuous wave laser (Coherent UK). Fluorescence lifetime images were obtained using a modified confocal microscopy apparatus, constructed in
the Central Laser Facility, which has a Nikon eC1 scanhead. Laser light was focused through a x60 water immersion objective with an NA of 1.2 on an inverted Nikon microscope (TE2000-U). Emission was collected without descanning, by-passing the scanning system, and passed through a bandpass filter (BG39, Comar). The scan was operated in the normal mode and line, frame and pixel clock signals were generated and synchronized with an external fast micro-channel plate photomultiplier tube (Hamamatsu R3809U) used as the detector. These were linked via a time-correlated single photon counting (TCSPC) PC module SPC830 (Becker and Hickl). The set-up provided instrument quantum efficiencies of more than 50% with single photon detection capabilities. Steady-state grey-scale multiphoton images (8 bit, up to 256 x 256 pixels) are produced by binning all decay photons as a single channel. Emission lifetime images were obtained by analysing the decay at individual pixels using a single or double exponential curve fitting following some modification to the standard Becker-Hickl SPCIImage analysis software (B&H SPCIImage 2.94) to allow analysis of the microsecond decay domain. A thresholding function within the analysis software ensured that non-correlating photons leading to background noise arriving at the detector were not included in the analysis. The lifetime image data are presented without further image processing.
Appendices 1. Preliminary Investigations into the bioreduction of uranium by the Gram-positive bacteria, *Alkaliphilus Oremlandii* by optical spectroscopy and microscopy.

**Introduction**

The use of a deep geological disposal facility (GDF) to store nuclear waste has been considered by many countries across the world as the best way to deal with legacy radioactive waste.¹ The current design is based around a multi-barrier containment system including, vitrified waste, a steel canister, a cementitious or clay based back fill material such as bentonite plus the host geology and intrinsic microbacterial interactions contributing to the biogeochemistry of the surrounding area.² Due to the presence of this cement or clay, the pH of the surrounding area is expected to be slightly alkaline, up to pH 10.³ While considerable research into the behaviour of bacteria at neutral pH has been carried out, there is much less on alkaline bacterial behaviour, which are expected to be much more prevalent at high pH.

There are some strains of bacteria that have been shown to function at high pH.⁴ *Alkaliphilus oremlandii* (formally known as *Clostridium* sp.) is a strictly anaerobic gram-positive bacteria⁵ and was isolated from sediments of the Ohio River (Pittsburgh, USA). Gram-positive bacteria have a thicker peptidoglycan layer than gram-negative bacteria and hence retain the violet stain during gram staining. Gram-positive bacteria have a simpler outer membrane than Gram-negative bacteria that contain surface bound species such as cytochromes, which have been implicated in the bioreduction of metals.⁶ A lack of these cytochromes in Gram-positive bacteria has important implications on the mechanisms involved in the reduction of metals by Gram-positive bacteria, and indicates that other redox active species mediate the observed bioreduction process instead.

Arsenic is commonly found in the environment as either arsenate(V) or arsenite(III) and they are associated with the oxic and anoxic environments respectively.⁷ *Alkaliphilus oremlandii* is capable of anaerobic respiration at pH 8.4 using arsenic as the electron acceptor with many potential electron donors such as acetate, lactate or pyruvate.⁸ Fischer *et al* studied the reduction arsenate(V) to arsenite(III) by *Alkaliphilus Oremlandii* over the course of a 50 hour reduction.⁸ The ability of *Alkaliphilus oremlandii* to reduce arsenate(V)
to arsenite(III), which is a 2-electron reduction process, suggests that this bacteria may also be able to reduce uranyl(VI) to uranium(IV), also a 2-electron reduction, although this strain of bacteria has not yet been investigated for its uranyl(VI) reduction capability.

Luminescence spectroscopy is a highly sensitive technique that can be carried out in situ and in principle can be used to distinguish between the different oxidation states of uranium (here, the +VI and +IV oxidation states) using their differing emission profiles and luminescence lifetimes (typically, μs for uranyl(VI) and ns for uranium(IV)).

Uranyl(VI) emission is generally accepted as a ligand-to-metal charge transfer emission centred around ca. 520 nm while uranium(IV) emission typically arises due to intra f-f transitions, resulting in line like emission spectra corresponding to various relaxation pathways.

In this contribution, we report the spectroscopic analysis of the interaction of Alkaliphilus oremlandii with uranyl(VI) acetate for the first time, along with fluorescence microscopy to gain an insight into the interaction of uranium and the bacteria on a cellular level and to investigate the bioreduction mechanism in more detail.

**Experimental**

Cells were grown in DMZ growth medium (250 mL), with an inoculation volume of 10 mL for 3 days at 30 ºC.

All cell manipulations were carried out under an atmosphere of N₂. Cultures were harvested by centrifugations and washed twice in NaHCO₃ buffer (30 mM, pH 8.2, degassed with N₂-CO₂ 80:20 mix). Aliquots of the washed cell suspensions were added to a final optical density of 0.3, by use of a syringe fitted with a needle, to anaerobic bottles sealed with butyl rubber stoppers containing uranyl(VI) acetate (5mM) in NaHCO₃ buffer (30 mM, pH 8.2). 10 mM lactate solution as the electron donor was added to the cultures for all A. Oremlandii experiments.

Samples were removed from the culture at various time points, every hour for initial experiments followed by every day for subsequent ones, by use of a syringe fitted with a needle and filtered into a low temperature quartz EPR tube or placed onto a quartz slide and sealed with a cover slip for low temperature luminescence and microscopy studies respectively. Steady state emission spectra were recorded on an Edinburgh Instrument FP920 Phosphorescence Lifetime Spectrometer equipped with a 5 watt microsecond pulsed
xenon flash lamp and a red sensitive photomultiplier in peltier (air cooled) housing, (Hamamatsu R928P). Lifetime data were recorded following 420 nm excitation with a 5 Watt xenon flash lamp (Edinburgh Instruments), using multichannel scaling. The one photon-excitation FLIM-PLIM is a commercial system from Becker and Hickl with a 405 nm pulsed laser system. Fluorescence lifetime images were obtained using a modified confocal microscopy apparatus, constructed in the Central Laser Facility, which has a Nikon eC1 scanhead. Laser light was focused through a x60 water immersion objective with an NA of 1.2 on an inverted Nikon microscope (TE2000-U). Steady state spectra were recorded using an Ocean Optics USB2000+ spectrometer.

Results and Discussion

A reduction experiment containing uranyl(VI) acetate (5 mM) and lactate (10 mM) in a 30 mM bicarbonate buffer was carried out at pH 8.2. The concentration of uranyl(VI) in solution was monitored at low temperature in frozen solution (77 K) using luminescence spectroscopy, from T=0 to T=6 hours. As can be seen in Figure 1 below, there is very little reduction in the intensity of the uranyl(VI) emission peak over the course of the reduction and it was therefore stopped after 6 hours.

Figure 1: Luminescence spectra showing the decrease in uranyl(VI) emission over the course of 6 hours during a bioreduction of uranyl(VI) acetate (5mM) in a 30 mM bicarbonate buffer, at pH 8.2, following excitation at 420 nm.

The reduction of uranium by *Alkaliphilus oremlandii* may be a process that occurs over days rather than hours and therefore the reduction was monitored over a longer time scale.
Figure 2 below shows the concentration of uranyl(VI) in solution over a 6 day period, and as can be seen, the concentration of uranium does not decrease dramatically over the course of the experiment. There is a small decrease in the luminescence intensity of uranyl(VI) from day 0 to day 1 which could be due to the sorption of uranium onto the surface/cell wall of the bacteria. This is supported by liquid scintillation data which is used to monitor the concentration of uranium in solution. The total concentration of soluble uranium also decreases between day 0 and day 1, and can be attributed to sorption of uranyl(VI) onto the surface of the bacteria which would no longer be in solution and would therefore not be measured by the liquid scintillation method in agreement with the luminescence data. Monoexponential lifetimes of ca. 1000 μs remained constant throughout the course of the experiment.

Figure 2: Luminescence spectra showing the decrease in uranyl(VI) emission over the course of 6 days during a bioreduction of uranyl(VI) acetate (5mM) by in a 30 mM bicarbonate buffer at pH 8.2, following excitation at 420 nm.
Figure 3: Change in luminescence intensity (black) and liquid scintillation (blue) data for A. orelmandii over the course of 6 days with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor in a bicarbonate (30 mM) buffer at pH 8.2. (Normalised to the initial starting intensity).

The lack of appreciable reduction in this system could be due to many different factors such as cell viability as a very low yield of cells is obtained after a 3 day growth period, the presence of oxygen during sampling as the bacteria are very strict anaerobes, or potentially because the reduction was occurring on such a slow time scale that the effects could not be monitored within the time constraints of this work.

One-photon fluorescence confocal microscopy was utilised in an attempt to visualise the potential interaction between the bacteria and the uranium. As can be seen in Figure 4, the average luminescence lifetime of up to 1190 μs across the majority of the cell remains constant, suggesting homogeneity with respect to uranyl(VI) concentration and/or speciation across the surface of the cell. These lifetimes are slightly lower than those observed for Gram-negative strains Geobacter sulfurreducens and Shewanella oneidensis. Interestingly, there is an area surrounding each of the bacteria that exhibits shorter average lifetime values of 733 μs than those observed on the surface. This may well be due to the presence of lipopolysaccharides (LPS) on the outer membrane which are effective at sorbing uranyl(VI). This was also observed in S. oneidensis mutants that were lacking inner and outer membrane cytochromes but on a much smaller scale. These data imply that the majority of the uranyl(VI) is just surface sorbed to the bacterial cells and due to the slow timescale of bioreduction can be envisioned as a “snapshot of sorption” with the uranyl(VI) and the LPS forming a layer around the bacteria, with shorter uranyl(VI) lifetimes due to the interaction between the two species.
Figure 4: One-photon fluorescence lifetime image microscopy (FLIM) map of lifetime data for *A. oremlandii* at T=2 during a bioreduction with uranyl(VI) acetate (5 mM) and lactate (10 mM) as the electron donor (left) and a representative spectrum obtained for an individual bacterium (denoted a one FLIM map) following excitation at 420 nm.

The luminescence lifetimes obtained for these samples were bi-exponential with a short lived component of 392.07 μs and a much longer lived component of 2528.70 μs indicating two luminescent species are contributing to the overall image. The shorter lifetime may be uranyl(VI) that is interacting with the bacteria and the longer lifetime may be uranyl(VI) that is not interacting with the bacteria and is therefore not being quenched by any surface bound species. This is confirmed by the steady state spectrum obtained from the outer layer one of the bacterium within the sample. The spectrum shows a broad peak at ca. 520 nm indicative of uranyl(VI), with the lack of vibrational fine structure suggesting more than one species is present contributing to the overall spectrum.

The same pattern of lifetime distributions was observed at various time points of T=2 and T=24. This could suggest that reduction by these bacteria is not occurring, which would be consistent with bulk fluorescence techniques, but that a small amount is sorbed on to the outer surface of the bacteria before being released back into solution.
Conclusion

In summary, these preliminary results suggest that the bioreduction of uranium by the Gram-positive bacteria *Alkaliphilus oremlandii* may be occurring over a long timescale that could not be measured in this research due to time constraints. Initial luminescence spectroscopy results suggest there is a sorption step in the mechanism prior to any reduction during the first day, indicated by a decrease in uranyl(VI) concentration measured by luminescence spectroscopy and a decrease in uranium concentration in solution measured by liquid scintillation counting. Fluorescence microscopy also suggests a sorption step with fluorescence lifetime image microscopy (FLIM) maps suggesting a high degree of homogeneity across the surface of the bacteria with a lipopolysaccharide and uranyl(VI) ring forming on the outer surface of the bacteria. This is in contrast to the Gram-negative *Geobacter sulfurreducens* and *Shewanella onedensis* bacteria genotypes that undergo much faster bioreduction with uranyl(VI) and exhibit complex heterogeneity with respect to uranyl(VI) luminescence lifetimes. This implies that the lack of cytochromes in the Gram-negative species *A. oremlandii* greatly impedes the bioreduction process of uranyl(VI).

Future work will include experiments conducted over a longer timescale in an effort to follow the reduction to completion. Once the timescale of the reduction has been established, a variety of samples would be investigated using fluorescence microscopy to investigate the speciation of uranium throughout the course of the reduction, rather than just during the initial stages.

References.


