The Bacterial Biogenic Synthesis of Magnetic, Catalytic and Semiconducting Nanomaterials

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Engineering and Physical Sciences

2011

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Abstract

The University of Manchester
Jonathan William Haddon Fellowes
Doctor of Philosophy (PhD)
The bacterial biogenic synthesis of magnetic, catalytic and semiconducting nanomaterials
2011

The environmental, microbiological and technological aspects of selenium is explored with the aim of assessing and identifying microorganisms capable of interacting with Se in the environment and forming functional ‘bionanomaterials’. To determine the natural microbial response to high selenium concentrations, and to understand the role soil microorganisms play in transforming Se, a field site in Co. Meath, Ireland, was identified and sampled to determine the Se contents. Detailed examination of the soil profile showed toxic levels of Se up to 156ppm. The highest Se concentrations correlated with elevated concentrations of higher plant matter, inferring a phytoconcentration mechanism for Se within a post glacial fen, and Se was identified as a reduced organic species. Microcosm experiments were established to test whether the soil microbial community displayed increased resistance to Se. These revealed the Se present in the soil was recalcitrant to microbial degradation and Sevi enriched experiments were noted to cause drastic alterations in community structure, indicating elevated Se resistance was not widespread throughout the community. Despite this, amended Sevi was rapidly reduced to Se0, as determined by XAS. Selenium, and the group 16 element tellurium, also display physico-chemical properties that make them ideal for a range of industrial, chemical and technological applications, including sequestration of hazardous wastes and as metal chalcogenide semiconducting ‘quantum dots’. Se0 and Te0 bionanomaterials formed by ‘resting cell’ cultures of the model environmental isolate Geobacter sulfurreducens, despite low MIC values, were characterised and subsequently applied to the sequestration of Hg0, derived from Hg historically used to preserve herbarium specimens. This showed that the Hg can be sequestered by the Se0 bionanoparticles in the form of HgSe and demonstrated increased stability over abiotic counterparts.

Finally, the bacteria G. sulfurreducens, Shewanella oneidensis and Veillonella atypica were compared for SeIV reducing capabilities, and V. atypica was shown to be adept at the production of significant quantities of SeII utilising the electron shuttle AQDS. Biogenic SeII compared favourably with abiotic SeII solutions in the formation of metal selenide quantum dots, displaying increased particle growth control as shown via a novel, time resolved XAS technique. Bacterial polymeric substances are inferred in controlling SeII precursor stability. This research shows that bacteria represent an alternative, facile, ‘green’ synthetic method for the production of next-generation technological nanomaterials.
Declaration

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 or other institute of learning.
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About the author

The author graduated from the University of Manchester in 2007 with an undergraduate masters degree in Earth Science (MEarthSci), specialising in Geochemistry. During this time, the author spent two summers working within the Geomicrobiology group of the School of Earth, Atmospheric and Environmental Sciences, under the supervision of Professor Jon Lloyd, Dr Victoria Coker and Dr Richard Cutting. The summer research project was developed in to the author’s MEarthSci research project, which was subsequently published as Cutting et al 2009*. The author has been engaged in the research reported in this thesis from 2007 to present.

Acknowledgements

I always found it interesting that the first thing anybody would read of a PhD thesis would be the acknowledgements section; especially as it is usually the last part written, and written at a time when the author has been staring at nothing but Microsoft Word for the previous 12 months, which tends to give a somewhat unusual perspective on the world (including causing you to look for little red squiggles underlining words in newspapers and magazines).

Anyway, life prior to the onset of ‘thesitis’ would have been immeasurably more difficult had my wonderful girlfriend Caz (and her amazing beef and ale pies) not been involved, keeping me on the slightly unhinged side of sane for these long, long months. I’d also like to acknowledge my mother and brother, although I’m unsure which side of sanity they were trying to send me.

My eternal gratitude goes out to my three ever-suffering supervisors; Richard Pattrick, who regrets ever giving me his phone number, Jon Lloyd, who has been forced to relocate somewhere in deepest, darkest Staffordshire to get away, and Carolyn Pearce, who for the same reason has emigrated to the far side of the world: my thousand word emails can still reach you there, though!

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work with the XRD, Chris Muryn for his help with the SAXS instrument in Chemistry, John Charnock for his help with XAS work on our numerous trips to synchrotrons dotted around the world, Paul Wincott for his help with XPS, Bart van Dongen and Wafa Al-Lawati for their work with the GC-MS, Cian Condon of Teagasc, Ireland, Garrett A. Fleming for dragging him through numerous fields in Co. Meath until we finally found the one he worked on nearly 50 years ago, and David Green for his help working in (and giving me a tour of) the Manchester Museum.

I also want to acknowledge the financial support of NERC, UK for paying for me to do this work, and numerous institutions including the (now retired) CCLRC Daresbury synchrotron, Warrington, UK, the ESRF, Grenoble, France, the Diamond Light Source, Oxfordshire, UK and the Molecular Foundry, Berkeley, California.

Finally, I promised myself that I would only include one (intentional) quote of the late, great Douglas Adams in this work, and I’ve decided that the following, despite originally being about the character Zaphod Beeblebrox, is a pretty apt description of the scientific method in general;

“He attacked everything in life with a mix of extraordinary genius and naive incompetence, and it was often difficult to tell which was which.”
Thesis approach

This thesis has been written using the alternative (scientific paper) format guidelines. The alternative format has been chosen due to the necessity for the rapid investigation and publication of research in an active, swiftly advancing area, and complements the style of research undertaken in the cross-disciplinary fields of biogeochemistry and bionanotechnology.
**Thesis structure and status of manuscripts**

The structure of this thesis, and where appropriate the status of the manuscripts and collaborator contributions, is summarised below;

**Chapter 1 – Introduction and literature review**

Section 1 – introduction and aims  
Section 2 – geochemistry of selenium and tellurium  
Section 3 – microbial interactions with selenium and tellurium  
Section 4 – technological applications of selenium and tellurium

**Chapter 2 – Review book chapter**

“Nanoparticles formed from microbial oxyanion reduction of toxic group 15 and group 16 metalloids”

_Published work_


_Author contributions_

_C. I. Pearce_ – principle author, experimental work on _Geobacter sulfurreducens_ and _Veillonella atypica_ with Se and Te, SEM/TEM analysis, microcosm set up, field work.  
_S. M. Baesman_ – manuscript reviewer, experimental work on _Bacillus selenitireducens, Sulfurospirillum barnesii_ and _Bacillus beveridgei_ strain MLTeJB with Se and Te, SEM/TEM analysis, microcosm set up, field work.  
_J. S. Blum_ – manuscript reviewer, experimental work on _Citrobacter_ Strain TSA-1 and the Searles Lake mixed antibiotic-fed enrichment culture (Ab-1) with AsS,
SEM/TEM analysis, microcosm set up, field work. J. W. Fellowes – manuscript author and reviewer, experimental work on Geobacter sulfurreducens with Te, SEM/TEM analysis, microcosm set up. R. S. Oremland – manuscript author and reviewer.

Acknowledged contributors


Chapter 3 - Methodology

Section 1 – electron optics
Section 2 – x-ray techniques
Section 3 – ion chromatography
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Section 5 – spectrophotometry
Section 6 – microbiological techniques

Chapter 4 – Research article 1

“Microbial Selenium Transformations in Seleniferous Soils”

Manuscript in preparation


Author contributions

J. W. Fellowes – principal author, soil core acquisition at field site, anaerobic manipulation of soil cores, XRF sample preparation and data interpretation,
assistant in XAS data collection, XANES data analysis and interpretation, lipid biomarker interpretation, Fe determination of microcosms, IC and ICP data interpretation of microcosm solutions, TRFLP statistical analysis and interpretation.

**R. A. D. Pattrick** – manuscript reviewer, initial identification of field sites, field work, microcosm set-up. **C. Boothman** – microbial community DNA extraction and TRFLP digest reactions. **W. M. Al-Lawati** – lipid biomarker experimental work. **B. E. van Dongen** – assistance with lipid biomarker analysis, editing of manuscript. **J. M. Charnock** – assistance with XAS data acquisition, XANES background subtraction and EXAFS modelling. **J. R. Lloyd** – manuscript reviewer, supervision of project, assistance with data interpretation. **K. Kvashnina** – local contact for ESRF beamtime allocation. **C. I. Pearce** – primary supervisor, manuscript reviewer, initial identification of field sites, field work, microcosm set-up, ESRF XAS beamtime principle investigator.

**Acknowledged contributors**

**G. A. Fleming** and **C. Condon** – assistance in identifying the Co. Meath field site and in field work. **A. Bewsher** – IC analysis. **P. Lythgoe** – ICP-AES, ICP-MS and XRF analyses. **J. Waters** – XRD analysis.

**Chapter 5 – Research article 2**

“Reductive biosynthesis of elemental selenium and tellurium nanoparticles by Geobacter sulfurreducens”

*Manuscript in preparation*


**Author contributions**

**J. W. Fellowes** – principle author, experimental work, SEM analysis, particle size analysis. **R. A. D. Pattrick** – manuscript reviewer. **J. R. Lloyd** – manuscript reviewer, data interpretation assistance. **C. I. Pearce** – primary supervisor, manuscript reviewer, TEM of Te⁰ precipitates.
Acknowledged contributors


Chapter 6 – Research article 3

“Use of biogenic and abiotic elemental selenium nanospheres to sequester elemental mercury released from mercury contaminated museum specimens”

Published work


Author contributions

J. W. Fellowes – principle author, sample acquisition, Hg$_0$ (air) determination, laboratory/experimental work, Se$_0$ characterisation, Se$_0$ filter preparation, SEM analysis, CVAAS analysis and interpretation, XAS analysis and interpretation.

R. A. D. Pattrick – manuscript reviewer, assistance with data interpretation.


Acknowledged contributors


Chapter 7 – Research article 4
“Investigating different mechanisms for biogenic selenite transformations: Geobacter sulfurreducens, Shewanella oneidensis and Veillonella atypica”

Published work


Author contributions


Acknowledged contributors


Chapter 8 – Research article 5

“Ex situ formation of metal selenide quantum dots using bacterially derived selenide precursors”
Manuscript in preparation


Author contributions

J. W. Fellowes – principle author, culturing of V. atypica, synthesis of GSH-CdSe quantum dots, UV/vis and photoluminescence analyses, assistant for ESRF XAS beamtime, XAS data manipulation and interpretation, particle size determination.

R. A. D. Patrick – manuscript reviewer, assistance with ESRF and DLS XAS beamtime allocation. J. R. Lloyd – manuscript reviewer, assistance with protein analysis and interpretation. J. M. Charnock – assistance with ESRF and DLS XAS beamtime allocation, assistance with XAS data interpretation. V. S. Coker – assistance with ESRF and DLS XAS beamtime allocation. J. F. W. Mosselmans – DLS XAS beamtime (S analysis) local contact. T.-C. Weng – ESRF XAS beamtime (Se analysis) local contact. C. I. Pearce – primary supervisor, manuscript reviewer, synthesis of abiotic HSe⁺, synthesis of βME-ZnSe, ESRF XAS beamtime principle investigator, protein analysis.

Acknowledged contributors


Chapter 9 – Conclusions and future work

Section 1 – Conclusions
Section 2 – Future work
Chapter 1

Introduction and literature review

Introduction and review of the current understanding of Se and Te geochemistry, biochemistry and technological applications
1 Introduction and project aims

The chalcogen metalloid selenium (Se) is an essential trace element for humans and many animals, but displays the narrowest concentration range between dietary deficiency (<40µg day⁻¹) and toxicity (>400µg day⁻¹) (58, 179). Se deficiency can result in diseases such as white muscle disease (in animals) and Keshan disease (in humans), whilst Se toxicity (‘selenosis’) has been reported in humans and animals (as ‘alkali disease’ and ‘blind staggers’) having consumed plants containing >5mg kg⁻¹ Se (58, 182). The bioavailability (and thus toxicity) of Se is dependent on the oxidation state; Se can be found in the environment as +VI, the oxyanion selenate; +IV, the oxyanion selenite; as a zero-valent, elemental form; as –II, selenide (48, 58, 121, 132), as well as a number of methylated compounds such as dimethylselenide and dimethyldiselenide (49, 101) and chalcogen-bearing amino-acids (and biochemical precursors and subsequent proteins) such as selenocysteine and selenomethionine (19, 76, 154, 185). The range of organo-selenium compounds observed in the environment highlight the degree to which soil microorganisms rework Se, a fact supported by a global Se geochemical cycle proposed by Shrift (148) which emphasises the dominant role microorganisms play. The principal mechanism by which Se enters the food chain is via uptake and incorporation into plant tissue, the extent of which is largely dependent on Se form.

The global distribution of Se in soils is heterogeneous, with most soils containing low Se values (0.01 – 2.0 mg Se kg⁻¹, global average 0.4mg Se kg⁻¹ (20, 59)). Amendment of agricultural soils in Se-poor areas, notably Finland (20, 52, 53, 58), has been reported, aiming to increase Se intake in human and animal populations. It is therefore important to understand the role soil microorganisms play in Se mobility to fully understand the fate and efficiency of these amendments.

Se-rich (‘seleniferous’) soils have also been reported around the world, and are commonly associated with Se-rich parent rocks from which the soils have formed – although not necessarily the rocks over which the soils lie. Several highly seleniferous soils have been reported, notably those in China (60, 182),
India (40-43), Ireland (55, 122) and the western United States (38, 39, 118, 131-133, 178), with concentrations in excess of 1200ppm Se reported (55). Consequently, cases of selenosis have also been reported in these areas; notably an outbreak of selenosis in Hubei Province, China, affecting ~50% of the 248 inhabitants (182), and the outbreak of acute selenosis within the Kesterson National Wildlife Refuge in the San Joaquin Valley, California, USA, culminating in the death or deformity of 64% of wildfowl embryos in 1983 (118, 131). In this case, Se (as the mobile selenate oxyanion, SeO$_4^{2-}$) originated from the weathering of seleniferous cretaceous shales, and was transported within agricultural drainage waters into local wetlands (38, 39, 118, 131-133, 178). The accumulation and evapoconcentration of Se and other metals within the wetlands resulted in the cases of Se poisoning reported (131), and highlights the requirement for the further development of a complete Se geochemical cycle, with a clear understanding of the dominant roles soil microorganisms play. Furthermore, a complete understanding of the biogeochemical interactions of soil microorganisms with Se-contaminated waste streams may allow for the development of novel bioremediation strategies (22, 89, 91, 92, 94, 103, 120).

Se and the closely related chalcogen tellurium (Te) also display physico-chemical properties that make them desirable for chemical, industrial and technological applications. The field of nanotechnology has the potential to revolutionise many aspects of every day life, having impacts ranging from medicine and drug delivery to next generation lasers and televisions. The traditional chemical routes toward the formation of nanomaterials have several disadvantages, specifically the requirement for toxic chemical precursors, hazardous high temperature conditions and significantly, high economic costs. Microbial precipitation of nanoscale crystals of technological interest offers an alternative, facile, ‘green’ synthesis technique, with research even suggesting that microorganisms may be capable of producing ‘bionanomaterials’ that are unique in structure and/or properties from chemically formed counterparts (121).
The dominant role of bacteria in the natural Se cycle and the increasing interest in chalcogen bionanomaterials has encouraged the exploration of the natural environment in search of bacteria that could be applied to the formation of novel, relevant nanomaterials. The scope of the natural world however dictates that research is required to focus the search for biotechnologically relevant bacteria, and to find links between noted geochemical conditions and possible exploitable biochemical mechanisms.

The principle hypothesis guiding this work is

“Microbial communities that are found within areas of naturally high Se concentrations will contain members that display resistance, detoxification or respiratory mechanisms that can be adapted to the development of novel bionanomaterials with technological, industrial and chemical applications.”

To this end, this research project aims to;

- Primarily, investigate the possibility of finding novel synthesis routes of technologically, industrially or chemically important chalcogen nanomaterials by environmentally isolated bacteria, with the aim of specifically targeting environmental areas where high chalcogens concentrations exist.
- Increase the understanding of selenium environmental geochemistry, and the role microorganisms play in enriching selenium in the environment (with specific reference to an identified seleniferous soil in County Meath, Ireland).
- Investigate the bacterial biogenic formation of chalcogen nanomaterials, and investigate their use in ‘real world’ scenarios.
- Investigate the bacterial biogenic synthesis of metal chalcogenide semiconducting ‘quantum dots’, and to make direct comparisons against abiotic counterparts to investigate the potential benefits and drawbacks of a bacterial ‘green’ formation route.
The following sections detail the current understanding of Se and Te in geochemical settings (section 2), known biochemical mechanisms (section 3) and finally the principles behind the technological applications of Se and Te (section 4), and form a basis upon which this research project extends.
2 Geochemistry of selenium and tellurium

2.1 Sources of selenium and tellurium

Selenium and tellurium initially enter into the environment due to the decomposition of parent rocks; the selenium and tellurium content of various rock types are listed in tables 2.1 and 2.2, respectively. The concentration of selenium in the lithosphere is estimated to be approximately 0.05ppm (49, 59, 129). Selenium in its most reduced form, selenide, has similar chemistry to the relatively common sulfide anion (average crustal abundance of 350ppm) due to their similar ionic radii (1.91Å and 1.84Å, respectively (50)), resulting in similar geochemical behaviour during petrogenesis. The selenium and sulphur content of igneous rocks correlates well \( r^2=0.96 \) (132) due to the stoichiometric substitution of \( \text{Se}^{2-} \) and \( \text{SeS}^{2-} \) into common sulphide-bearing minerals such as pyrite, pyrrhotite, chalcopyrite, arsenopyrite, galena and sphalerite (2, 99, 131). Selenium is also rarely found as metal selenide minerals with copper, iron, lead and mercury (17).

Table 2.1 shows the average selenium concentration of a range of intrusive and extrusive igneous rocks. The selenium concentration of plutonic rocks rarely exceeds the crustal abundance as selenium is lost as a volatile phase (as \( \text{H}_2\text{Se}, \text{SeO}_2 \)) during the early stage of magma fractionation, resulting in selenium depletion of igneous rocks relative to the magma source (17, 59). The decreasing selenium content of igneous rocks produced by fractional crystallisation coincides with an increase in silica concentrations; granitic bodies produced by these processes are typically below the average crustal abundance of selenium (table 2.1) (58, 99).

Volatilised and incompatible selenium is enriched in extrusive igneous rocks relative to average crustal concentrations (as shown in table 2.1), and is found as discrete mineral phases within hydrothermal deposits e.g. the pyrite isomorph ferroselite \( (\text{FeSe}_2) \) and challomenite \( (\text{CuSeO}_3.2\text{H}_2\text{O}) \) (49). Selenium within hydrothermal deposits such as the large seleniferous volcanic-hosted massive sulphide deposit in the Finlayson Lake District, Yukon, Canada is heterogeneously distributed, with concentrations up to 3420ppm (87).
<table>
<thead>
<tr>
<th>Parent Rock</th>
<th>Selenium (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Crustal Abundance</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Igneous Rocks</strong></td>
<td></td>
</tr>
<tr>
<td>Ultramafic</td>
<td>0.05</td>
</tr>
<tr>
<td>Mafic</td>
<td>0.05</td>
</tr>
<tr>
<td>Granitic</td>
<td>0.01 - 0.05</td>
</tr>
<tr>
<td>Volcanic Rocks</td>
<td>0.35</td>
</tr>
<tr>
<td>Volcanic Rocks (Hawaii)</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Volcanic Tuffs</td>
<td>9.15</td>
</tr>
<tr>
<td><strong>Sedimentary Rocks</strong></td>
<td></td>
</tr>
<tr>
<td>Marine Carbonate</td>
<td>0.17</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.03 - 0.08</td>
</tr>
<tr>
<td>Sandstone</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Californian Shales</td>
<td>1 - 675</td>
</tr>
<tr>
<td>Wyoming Shales</td>
<td>2.3 - 52</td>
</tr>
<tr>
<td>Finnish Shales</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Mudstones</td>
<td>0.1 - 1,500</td>
</tr>
<tr>
<td>Phosphatic Sediments</td>
<td>1 - 300</td>
</tr>
<tr>
<td>Coal (USA)</td>
<td>0.46 - 10.7</td>
</tr>
<tr>
<td>Coal (Australia)</td>
<td>0.21 - 2.5</td>
</tr>
<tr>
<td>Coal (Chinese)</td>
<td>&lt;6,500</td>
</tr>
<tr>
<td>Oil</td>
<td>0.01 - 1.4</td>
</tr>
<tr>
<td><strong>Ore Minerals</strong></td>
<td></td>
</tr>
<tr>
<td>Pyrite</td>
<td>&lt;10 – 300</td>
</tr>
<tr>
<td>Sphalerite</td>
<td>&lt;10 – 900</td>
</tr>
<tr>
<td>Chalcopyrite</td>
<td>&lt;10 – 3420</td>
</tr>
<tr>
<td>Pyrrhotite</td>
<td>&lt;10 – 63</td>
</tr>
<tr>
<td>Cinnabar</td>
<td>&lt;94000</td>
</tr>
</tbody>
</table>

*Table 2.1. Selenium content of typical and exemplary rocks.*

Sources: Plant (129), Layton-Matthews *et al* (87) and Berrow and Allan (17)
The selenium concentration of sedimentary rocks is highly variable, and depends on factors affecting the original sediments prior to diagenesis (see table 2.1). High concentrations of selenium occur in sediments near to volcanic centres, where particulate selenium may fall out or be washed out of the atmosphere and bound to the nearby sediments (17, 99). This results in selenium concentration variations between beds within a formation and also between portions of the same bed (17).

<table>
<thead>
<tr>
<th>Parent Rock</th>
<th>Tellurium (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Crustal Abundance</td>
<td>0.005</td>
</tr>
<tr>
<td><em>Igneous Rocks</em></td>
<td>0.001 – 0.006</td>
</tr>
<tr>
<td><em>Sedimentary Rocks</em></td>
<td>0.002 – 0.009</td>
</tr>
<tr>
<td>Greywacke and Shales</td>
<td>0.04 – 1</td>
</tr>
<tr>
<td><em>Pyrite Deposit</em></td>
<td></td>
</tr>
<tr>
<td>Pyrite</td>
<td>6 – 1350</td>
</tr>
<tr>
<td>Chalcopyrite</td>
<td>9 – 6600</td>
</tr>
<tr>
<td><em>Cu-Mo Deposit</em></td>
<td></td>
</tr>
<tr>
<td>Pyrite</td>
<td>5 – 510</td>
</tr>
<tr>
<td>Chalcopyrite</td>
<td>5 – 20</td>
</tr>
<tr>
<td><em>FeMn Nodules</em></td>
<td>3 – 205</td>
</tr>
</tbody>
</table>

*Table 2.2. Tellurium content of rocks and mineral deposits*

Sources: Hein *et al* (68), Jovic (78), Reimann and de Caritat (137), Vlassov (171), Ward *et al* (176), Wedepohl (177).

In the absence of volcanic activity, selenium concentrates in clay and iron rich rocks due to the strong adsorption of selenium to these materials (99).
Selenium is also retained in organic forms in phosphatic and organic rich rocks, most notably black shales. Owing to the strong correlation of selenium and organic carbon, fossil fuels such as coal and oil can contain a high amount of selenium. Crude oil from the USA contains up to 0.17ppm of selenium, and enrichments of two orders of magnitude (<3.36ppm) have been reported for selenium in coals originating from the USA (128).

Seleniferous rocks of the western United States are amongst the most heavily researched selenium-rich rocks due to several incidences of selenium poisoning amongst wildfowl (118, 131). Late cretaceous pyritic, diatomaceous and foraminiferal black shales contained within the Panoche and Moreno formations exposed in western California contain high concentrations of selenium, with an exposure of the Moreno formation 19km south of Tracy, CA containing 28ppm Se (132, 133). The seleniferous Pierre and Niobrara formations contain black shales that are widespread across the central United States and have a high selenium concentration that exceeds 100ppm (Summarised in 132). Seleniferous rocks outcrop in other areas around the world such as Ireland and India, and are often accompanied by occurrences of selenium toxicity in wildfowl, cattle and the local population (28, 29, 42, 43, 144).

Tellurium is much rarer than selenium, with an estimated lithospheric concentration of ~0.002 - 0.005ppm (30, 49, 50). As with selenium, tellurium is a chalcophile element and can be incorporated into sulfide minerals via the stoichiometric substitution of the Te$^{2-}$ (ionic radius of 2.21Å) or TeS$^{2-}$ dianions for S$^{2-}$ (ionic radius of 1.84Å). Tellurium is also found as distinct mineral phases (2, 78, 146), typically as gold and/or silver telluride minerals such as hessite (Ag$_2$Te), Sylvanite (Au$_4$AgTe$_4$), Krennerite (Au$_4$AgTe$_2$) and calaverite (AuTe$_2$) (123, 146, 185). Table 2.2 shows the estimated average abundance of tellurium in various types of rock and mineral deposits. In silicate minerals, the abundance of tellurium generally lies below detection limits for most techniques, however tellurium can be concentrated into epithermal and mesothermal vein deposits as well as into marine shale and greywacke deposits (146). Low concentrations of tellurium are also present as telluride in hydrothermal deposits (54, 77). Rare
examples of areas with high telluride content include the gold-silver-telluride deposits of the Emperor Gold Mine, Fiji (5), the mesothermal tellurium rich deposits of the ‘Golden Mile’, near Kalgoorlie, Western Australia (147), the epithermal gold-telluride minerals of the Kochbulak deposits in Uzbekistan (84) and the exotic telluride bearing minerals such as telargpalite ([Pd,Ag]_3[Te,Bi,Pb]) contained within the orthomagmatic Oktyabrsky deposits near the city of Norilsk, Siberia (83).

The occurrence of tellurium in sedimentary rocks is rare, owing to the low concentration of tellurium in the lithosphere, with the exception of iron-manganese nodules situated on the sea floor. Geochemical analysis of 12 nodules obtained from the floor of the Pacific and Indian Oceans has shown that tellurium is present in elevated concentrations of up to 125ppm (86), and further analysis of nodules obtained from the Atlantic Ocean contained 205ppm (69). Tellurium within these iron-manganese nodules is estimated to be concentrated to over 50,000 times the average crustal abundance, which is the highest enrichment of any element within these crusts (69).

2.2 Selenium and tellurium in the environment

The chemical similarities that lead to the coupling of selenium and sulfur in lithospheric processes do not carry through in to the oxic, aqueous environments of the biosphere. Environmental processes lead to the discrimination of sulfur and selenium owing to the differences in reduction potentials, resulting in sulfur readily becoming oxidised to the mobile sulfate phase, whilst the oxidation of elemental selenium to selenite is retarded due to the lanthanide-like contraction of the atomic radius indicative of elements succeeding the transition metals in the periodic table (17, 149).

The geochemical conditions under which seleniferous rocks are weathered determine the oxidation state of the selenium produced (99, 132). Under acidic/reducing conditions the selenite oxyanion (SeO_3^{2-}) is produced; basic, oxidising conditions cause the complete oxidation of selenium to the selenate oxyanion, SeO_4^{2-} (See also figure 2.1) (99, 132). At circumneutral pH, calculations made by Dhillon and Dhillon (41) specify that at selenium concentrations of 300µg l^-1,
elemental selenium would be the dominant form at an Eh < +0.27 mV, with aqueous species existing at > +0.27 mV. Even in environments where selenate is the thermodynamically favourable form, the transition from selenium to selenite is faster than the further oxidation to selenate (48, 101, 131, 154, 170). Selenate oxyanions are more mobile than selenite in the environment, indicating that total selenium is most mobile under oxidised, high pH conditions (17), and represent up to 84% of the selenium from the igneous parent rock (106).

Figure 2.1. Selenium Eh vs pH diagram. (After Johnson et al 74)

The average global soil selenium concentration is estimated to be 0.4 ppm, with sporadic high selenium concentrations up to 1200 ppm (as shown in table 2.3) (59). The average soil in the UK ranges between <0.01 to 4.7 ppm (59), and selenium is known to be particularly abundant in ferricrete and laterite soils (99).
The weathering of seleniferous rocks may lead to the release of selenium into groundwater aquifers. The average selenium concentration of groundwater is considered to be low at 0.2µg l\(^{-1}\) (117), however concentrations as high as 1300µg l\(^{-1}\) have been reported in shallow wells within the catchment area of the Colorado River, Utah (51), and up to 1000µg l\(^{-1}\) in aquifers in Montana, USA (129) (table 2.4).

The dominant selenium species present within an aquifer is largely controlled by the reduction potential, with the mobile selenate oxyanion common in oxidising conditions and the reduced, immobile forms such as elemental selenium and selenide salts present under reducing conditions (figure 2.1) (129). The dominant species present changes with depth, as the reduction potential within the groundwater decreases, and this is represented by the reduction in selenium mobility with depth (41). The selenium speciation may also change along the course of an aquifer, as indicated by the fluctuations in selenium concentration within the aquifer of the Triassic sandstone of the East Midlands, UK, which ranges from 0.86µg l\(^{-1}\) in seleniferous areas down to 0.06µg l\(^{-1}\) beyond the redox boundary (150). The selenium concentration of aquifers has been repeatedly noted to correlate well with the salinity of groundwater (3, 4, 38, 115, 129, 150, 151), and is thought to be connected to the evapoconcentration of selenium and possible effects of irrigation waters leaching soluble soil salts (129).

Selenium that is exposed at the surface may be mobilised in freshwater rivers and lakes. Information on the average concentration of selenium in surface freshwater environments is limited, however the suggested average is 60ng l\(^{-1}\) selenium, with only around 15% of this present as dissolved aqueous species; the remaining 85% being present as particulates (117). The concentration of selenium in river waters and lakes is heavily dependent on the geology and soil geochemistry of the watershed (139).
<table>
<thead>
<tr>
<th>Soil Type</th>
<th>Selenium (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average world soil</td>
<td>0.4</td>
</tr>
<tr>
<td>Average seleniferous soil</td>
<td>&lt;5000</td>
</tr>
<tr>
<td>Average USA soil</td>
<td>&lt;0.1-4.3</td>
</tr>
<tr>
<td>Average USA seleniferous soil</td>
<td>1-10</td>
</tr>
<tr>
<td>Average UK soil</td>
<td>&lt;0.01-4.7</td>
</tr>
<tr>
<td>Average Irish seleniferous soil</td>
<td>1-1200</td>
</tr>
<tr>
<td>Chinese average soil</td>
<td>0.02-3.8</td>
</tr>
<tr>
<td>Chinese average seleniferous soil</td>
<td>1.49-59</td>
</tr>
<tr>
<td>Chinese average Se-deficient soil</td>
<td>0.004-0.48</td>
</tr>
<tr>
<td>Finish average soil</td>
<td>0.005-1.25</td>
</tr>
<tr>
<td>Indian seleniferous soil</td>
<td>1-20</td>
</tr>
<tr>
<td>Indian Se-deficient soil</td>
<td>0.025-0.71</td>
</tr>
<tr>
<td>Laterite soils</td>
<td>0.3 – 0.6</td>
</tr>
<tr>
<td>Ferricrete soils</td>
<td>0.5 – 0.8</td>
</tr>
<tr>
<td>Soils over metabasalts</td>
<td>0.7 – 0.8</td>
</tr>
<tr>
<td>Soils over ultramafics</td>
<td>0.5 – 0.6</td>
</tr>
<tr>
<td>Soils over granites</td>
<td>0.02</td>
</tr>
<tr>
<td>Sandy soils over granites</td>
<td>0.05</td>
</tr>
<tr>
<td>Saprolite</td>
<td>0.01 – 0.3</td>
</tr>
</tbody>
</table>

Table 2.3. Selenium concentrations in soils around the world.

Sources: Malisa (99), Plant (129).
<table>
<thead>
<tr>
<th>Groundwater Location</th>
<th>Total dissolved Se (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Global Shallow Groundwater</td>
<td>0.2</td>
</tr>
<tr>
<td>Argentina</td>
<td>48 – 67</td>
</tr>
<tr>
<td>Australia</td>
<td>0.008 – 0.33</td>
</tr>
<tr>
<td>Belgium</td>
<td>&lt;0.06 – 1.33</td>
</tr>
<tr>
<td>France</td>
<td>&lt;5 – 75</td>
</tr>
<tr>
<td>Israel</td>
<td>0.9 – 27</td>
</tr>
<tr>
<td>Italy</td>
<td>&lt;0.002 – 1.94</td>
</tr>
</tbody>
</table>

**Specific Localities**

- Colorado River Catchment, Utah <1300
- Coast Range Aquifer, San Joaquin <1 - 2000
- Sierra Nevada Aquifer, San Joaquin <1
- Valley, CA, USA
- Hoshiarpur and Nawanshahar Districts, India 0.25 – 69.5
- Montana, USA 1000
- Pyrenees, S.France 2.36
- Soan-Sakesar Aquifer, Pakistan 62

*Table 2.4. Selenium content of groundwaters from around the world. Sources: Berrow and Allan (17), Dhillon and Dhillon (41), Engberg (51), Nriagu (117), Plant *et al* (129), Robberecht and Vangrieken (139).

The oceans are a much larger reservoir for selenium than freshwater sources; the combined mass of selenium in the oceans is estimated to be approximately $2.3 \times 10^8$ tonnes (117). The marine environment is divided into two main compartments with respect to the selenium concentration; the upper-most layer extends down from the surface to 75m depth, and has an average dissolved selenium concentration of 30ng l$^{-1}$, with a total selenium mass of
8.4×10^5 tonnes and the lower layer extends down from 75m to the ocean floor and contains around 1.3×10^8 tonnes of dissolved selenium, with an average concentration of 95ng l⁻¹ (31, 117). The residence time of selenium in surface waters has been calculated at 70 years, with rapid loss of selenium to the atmosphere as volatile selenium compounds as a result of biological reactions (31, 57, 117). The residence time of selenite in the lower reservoir is estimated to be 1150 years, and a marine selenium biogeochemical cycle proposed by Cutter and Bruland (31) suggests that selenite is the product of the oxidation of organic selenium detritus from the upper layer, in addition to selenate present due to the cycling of surface waters into the deep ocean. Mixing between these two reservoirs is notably one sided, with 4400 tonnes yr⁻¹ being delivered down to the deeper layer as mostly selenide, and the return of only 600 tonnes yr⁻¹ into the surface waters (31, 117). Selenium can also be found in the oceans as suspended particulates in the surface layer (3mg l⁻¹) and in pore water in ocean sediments (0.3µg l⁻¹) (31, 117).

The atmosphere represents an important transportation mechanism for volatile selenium compounds. Haygarth et al. (67) noted that the selenium concentration in coastal regions is enriched due to the transportation and subsequent deposition of ocean-derived volatile selenium. Modelling of the selenium concentration is thus divided into the atmosphere above the land and the atmosphere above the oceans. Emission over land represents only 15% of the total selenium emission from natural sources, estimated to be approximately 8400 tonnes yr⁻¹, with over 80% being emitted by natural sources into the atmosphere above the oceans (117). The dominant natural selenium input into the atmosphere above land is the release of volatile selenium species and particulates by volcanic activity (320 tonnes yr⁻¹) and volatile selenium products of plants and soil microorganisms (1200 tonnes yr⁻¹) (74, 99, 117). The primary source of selenium in the atmosphere above the oceans is from the biological volatilisation of selenium compounds by phytoplankton, and this can fluctuate seasonally due to changes in population as noted by Amouroux et al. (7).
Precipitation is thought to play a major role in the deposition of selenium from the atmosphere (17, 41). Particulate aerosol forms of selenium, commonly associated with volcanic emissions, are especially susceptible to being removed by rainfall and generally do not occur substantial distances from their source (17). The range of selenium concentrations in rainwater varies dramatically from <0.001 up to 2.5µg l⁻¹ (41, 140), dependent on local anthropogenic emissions, volcanic and biological activity. The calculated rate of selenium deposition from the atmosphere is around 15000 tonnes yr⁻¹, which has been used to calculate the residence time of selenium in the atmosphere to be 45 days (117). The long residence time of selenium in the atmosphere, relative to pollutant metallic particulates, suggests that selenium is present in the atmosphere mostly in volatilised form (41, 117).

The chemical breakdown of tellurium rich rocks typically leads to the liberation of the tellurite oxyanion (TeO₃²⁻) or telluric acid (Te(OH)₄), both of which are mildly soluble and therefore mobile within the environment. TeIV is preferentially scavenged by iron oxyhydroxides, and leads to the gradual oxidation to TeVI as H₅TeO₆⁻ (69, 78, 116). Owing to the low lithospheric concentration of Te, the occurrence of Te in the environment is rare.

In conclusion, the major factor governing total selenium concentration in the environment is the under-lying rock-type. Areas with high selenium content occur where the soils are derived from seleniferous rocks, such as occurs in the central valley of California, USA (133). Here, the selenium content of soils is further increased by the evapoconcentration of seleniferous waters in irrigation drainage ponds (133). The selenium concentration of soils can be further amended by the deposition of volatile selenium from the atmosphere as a result of biological activity from the oceans or as a result of local volcanic activity. As the tellurium content of rocks is relatively low, its occurrence in the environment is rare.
3 Microbial interactions with selenium and tellurium

A wide range of microorganisms, both environmental and clinical isolates, are capable of interacting with selenium and/or tellurium (table 3.1), whether oxidising, reducing or methylating for respiration or detoxification purposes, or to maintain redox poise. Shrift (148) suggests that bacteria and other microorganisms are the main driving force behind the cycling of selenium between reservoirs, and has lead to the description of a selenium cycle in nature (49, 90, 154).

To date, no environmental cycle has been developed for tellurium owing to the low environmental concentration, although it has been reported for over a century that bacteria can reduce tellurite to elemental tellurium (81) and can methylate tellurium oxyanions (15, 56). The microbial reduction of selenium and tellurium oxyanions to elemental nanoparticles or aqueous selenide/telluride ions due to either detoxification or respiration mechanisms is of particular interest to this research project due to technological, industrial and chemical applications of these materials.

3.1 Toxicity and detoxification of selenium and tellurium

Selenium is a toxic non-metal, forming stable, long-lived sulphur complexes that disrupt thiol intracellular biochemistry (185). Despite toxicity at higher concentrations, selenium is known to be an essential trace element and occurs in the amino acids selenocysteine and selenomethionine, which are incorporated into a range of proteins including glutathione peroxidise and formate dehydrogenase (14, 19, 154, 185). Several mechanisms of selenium resistance have been described and rely on the reduction of the soluble, mobile selenate and selenite oxyanions to insoluble elemental selenium or volatile methylselenide phases and via the regulation of redox poise within the bacterial cell (102, 105, 154, 156).

Tellurium is 100-1000 times more toxic than selenium, and no biochemical use for tellurium has been described (23, 146). Despite significant quantities of research regarding Te detoxification mechanisms, principally involving
<table>
<thead>
<tr>
<th><strong>Bacteria</strong></th>
<th><strong>Starting Material</strong></th>
<th><strong>End Product</strong></th>
<th><strong>Notes</strong></th>
<th><strong>Reference</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas stutzeri</em></td>
<td>Se&lt;sup&gt;VI&lt;/sup&gt;</td>
<td>Se&lt;sup&gt;0&lt;/sup&gt;</td>
<td></td>
<td>(96)</td>
</tr>
<tr>
<td><em>Sulfurospirillum barnesii</em></td>
<td>Se&lt;sup&gt;VI&lt;/sup&gt;</td>
<td>Se&lt;sup&gt;0&lt;/sup&gt;</td>
<td>Isolated</td>
<td>(155)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>freshwater drainage, Nevada</td>
<td></td>
</tr>
<tr>
<td><em>Thauera selenatis</em></td>
<td>Se&lt;sup&gt;VI&lt;/sup&gt;</td>
<td>Se&lt;sup&gt;IV&lt;/sup&gt;(Se&lt;sup&gt;0&lt;/sup&gt; in presence of nitrate)</td>
<td></td>
<td>(98)</td>
</tr>
<tr>
<td><em>Bacillus selenitireducens</em></td>
<td>Se&lt;sup&gt;IV&lt;/sup&gt;</td>
<td>Se&lt;sup&gt;0&lt;/sup&gt;</td>
<td>Isolated</td>
<td>(18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mono lake, CA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(18)</td>
</tr>
<tr>
<td><em>Bacillus arsenicoselenatis</em></td>
<td>Se&lt;sup&gt;VI&lt;/sup&gt;</td>
<td>Se&lt;sup&gt;IV&lt;/sup&gt;</td>
<td></td>
<td>(18)</td>
</tr>
<tr>
<td><em>Rhodospirillum rubrum</em></td>
<td>Se&lt;sup&gt;IV&lt;/sup&gt;</td>
<td>Se&lt;sup&gt;0&lt;/sup&gt;</td>
<td></td>
<td>(80)</td>
</tr>
<tr>
<td><em>Bacillus selenatarsenatis</em></td>
<td>Se&lt;sup&gt;VI&lt;/sup&gt;</td>
<td>Se&lt;sup&gt;0&lt;/sup&gt;</td>
<td></td>
<td>(181)</td>
</tr>
<tr>
<td><em>Thiobacillus ASN-1</em></td>
<td>Se&lt;sup&gt;0&lt;/sup&gt;</td>
<td>Se&lt;sup&gt;VI&lt;/sup&gt;</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td><em>Leptothrix MnB1</em></td>
<td>Se&lt;sup&gt;0&lt;/sup&gt;</td>
<td>Se&lt;sup&gt;VI&lt;/sup&gt;</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td><em>Acidithiobacillus ferrooxidans</em></td>
<td>Se&lt;sup&gt;-II&lt;/sup&gt;</td>
<td>Se&lt;sup&gt;0&lt;/sup&gt;</td>
<td></td>
<td>(165)</td>
</tr>
<tr>
<td><em>Pyrobaculum arsenaticum</em></td>
<td>Se&lt;sup&gt;VI&lt;/sup&gt;</td>
<td>Se&lt;sup&gt;0&lt;/sup&gt;</td>
<td></td>
<td>(73)</td>
</tr>
<tr>
<td><em>Pyrobaculum aerophilum</em></td>
<td>Se&lt;sup&gt;VI&lt;/sup&gt;</td>
<td>Se&lt;sup&gt;0&lt;/sup&gt;</td>
<td></td>
<td>(73)</td>
</tr>
<tr>
<td><em>Methanococcus</em></td>
<td>Se&lt;sup&gt;IV&lt;/sup&gt;</td>
<td>Org-Se</td>
<td></td>
<td>(76)</td>
</tr>
<tr>
<td>Microorganism</td>
<td>Selenium Form(s)</td>
<td>Tellurium Form(s)</td>
<td>Location</td>
<td>Reference(s)</td>
</tr>
<tr>
<td>----------------------------</td>
<td>------------------</td>
<td>-------------------</td>
<td>---------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Shewanella oneidensis</td>
<td>Se$^{IV}$, Se$^{0}$, Se$^{2-}$</td>
<td>Extracellular</td>
<td>n/a</td>
<td>(82, 124)</td>
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<tr>
<td>Corynebacterium</td>
<td>Se$^{0}$, Se$^{IV}$, Se$^{VI}$</td>
<td>Methyl-Se</td>
<td>n/a</td>
<td>(46)</td>
</tr>
<tr>
<td>Veillonella atypica</td>
<td>Se$^{IV}$</td>
<td>Se$^{II}$</td>
<td>Clinical isolate, biphasic reduction</td>
<td>(124)</td>
</tr>
<tr>
<td>Geobacter sulfurreducens</td>
<td>Se$^{IV}$</td>
<td>Se$^{II}$</td>
<td>n/a</td>
<td>(124)</td>
</tr>
<tr>
<td>Bacillus selenitireducens</td>
<td>Te$^{IV}$</td>
<td>Te$^{0}$</td>
<td>Internal Te$^{0}$ nanorods, external Te$^{0}$ ‘rosettes’</td>
<td>(10)</td>
</tr>
<tr>
<td>Sulfurospirillum barnesii</td>
<td>Te$^{VI}$</td>
<td>Te$^{0}$</td>
<td>External and internal Te$^{0}$ nanospheres</td>
<td>(10)</td>
</tr>
<tr>
<td>Desulfovibrio desulfuricans</td>
<td>Te$^{IV}$</td>
<td>Te$^{0}$</td>
<td>n/a</td>
<td>(95)</td>
</tr>
<tr>
<td>Shewanella oneidensis</td>
<td>Te$^{IV}$</td>
<td>Te$^{0}$</td>
<td>Intracellular (cytoplasmic) Te$^{0}$ ‘needles’</td>
<td>(82)</td>
</tr>
</tbody>
</table>

Table 3.1. Examples of microorganisms known to interact with selenium and tellurium.

Escherichia coli, the mechanisms of Te toxicity and detoxification are still poorly known (23). A model for the mechanism of tellurite toxicity described by Turner et al (168) states tellurite oxyanions enter a cell via uptake by a phosphate transport system and are subsequently reduced to elemental tellurium by glutathione or other cytoplasmic reduced thiols. Reactive oxygen species (O$_2^-$)
produced by the reduction of tellurite is then acted upon by superoxide dismutase, the deficiency of which allows for the oxidation of cellular thiols leading to the termination of DNA and protein synthesis (127, 160, 168). A second mechanism of toxicity is thought to be analogous to that of selenium, whereby tellurium is incorporated into thiol groups in place of sulphur (161, 174, 185).

Microbial tellurite resistance has been reported in a wide range of bacteria, with the genes determining this resistance present on plasmids and within the genomes of a wide range of unrelated bacteria (161). Furthermore, tellurite resistance (Te') mechanisms also appear to be unique, indicating that despite the low abundance of tellurite in the environment, several mechanisms evolved separately (185). There are five unique Te' genes known (table 3.2).

<table>
<thead>
<tr>
<th>Te' Determinant</th>
<th>Suggested MIC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ter</td>
<td>1024 µg ml⁻¹</td>
<td>(163)</td>
</tr>
<tr>
<td>tehAB</td>
<td>128 µg ml⁻¹</td>
<td>(174)</td>
</tr>
<tr>
<td>kilAtelAB/klaABtelB</td>
<td>256 µg ml⁻¹</td>
<td>(162)</td>
</tr>
<tr>
<td>tpmT</td>
<td>256 µg ml⁻¹</td>
<td>(27)</td>
</tr>
<tr>
<td>cysM/cysK</td>
<td>&gt;1000 µg ml⁻¹</td>
<td>(6)</td>
</tr>
</tbody>
</table>

*Table 3.2. Te' determinants and recorded MICs (Compiled from Zannoni et al 185)*

The mechanism of bacterial tellurite resistance is by the reduction of the soluble tellurite oxyanion to the insoluble elemental tellurium form, and can be noted by the black discolouration of tellurite-resistant colonies (161, 167). Gram negative bacteria are known to be particularly sensitive to tellurite and tellurate concentrations (185).

There appears to be some overlap between tellurite/tellurate and selenite/selenate resistance genes. Work by Cournoyer et al (27) has lead to the isolation of the selenite resistance gene *tpmT*, which works by producing methyl selenite compounds as well as producing methylated tellurite compounds. The
ubiE gene isolated from Geobacillus stearothermophilus also conveys both tellurate and selenate and selenite resistance by methylating the reduced products of these materials (9, 157).

3.2. Microbial respiration of selenium and tellurium

The reduction of selenium oxyanions by microorganisms during respiration is recognised to be an important factor in the cycling of selenium in the environment (90, 121, 148). The reduction of selenate and selenite as a terminal electron acceptor is energetically favourable, with the free energy of the reduction of HSeO₃⁻ coupled with the oxidation of H₂ yielding -8.93 kcal mol⁻¹ e⁻¹ (156). Selenium oxyanion reduction occurs in a wide range of bacteria, including representatives of the Wolinella, Pseudomonas, Sulphurospirillum, Enterobacter, Thaurea, Bacillus, Shewanella and Citrobacter genera.

The various mechanisms of selenium reduction by bacteria have been covered by Zannoni et al (185), and so will only be briefly described here. There are four pathways through which the reduction of selenium oxyanions may occur;

(i) a painter-type reaction, in which the selenium oxyanions are reduced to elemental selenium by glutathione via several intermediates such as selenodiglutathione and glucothioselenol (156);

(ii) oxyanion reduction by cytosolic and periplasmic oxidoreductases. It also suspected that the nitrate/nitrite and sulphate/sulphite reductases may participate in the metabolic reduction of selenium oxyanions;

(iii) abiotic reactions with metabolic products e.g. during the reduction of sulphate to sulphide, the sulphide can react with selenite to produce elemental selenium and sulphur (72);

(iv) the production of elemental selenium by a reaction with the biologically derived chelating agent pyridine-2,6-bisthiocarboxylic acid (PDTC), produced by Pseudomonas stutzeri and Pseudomonas putida, capable of binding to a wide range of metals, lanthanides and actinides (26).
Many of the mechanisms thought to enable microbial growth utilising tellurite as a terminal electron acceptor are similar to those previously stated for selenium owing to the very similar chemical nature of these compounds. Work published by Trutko et al (167) infers that two *Pseudomonas aeruginosa* strains (PAO ML4262 and PAO ML4262[pBS10]) and *Rhodobacter sphaeroides* (105) utilise c-type cytochromes and cytochrome oxidases during respiration on tellurite, as opposed to the quinol oxidase system known to be involved in tellurite respiration in *Escherichia coli*.

Microbial respiration of tellurate has recently been reported in bacteria isolated from hydrothermal vents in the eastern Pacific Ocean (30), and work by Baesman et al (10) has shown that *Sulfurospirillum barnesii* and *Bacillus selenitireducens* are capable of respiration using tellurate and tellurite oxyanions coupled to the oxidation of lactate.

### 3.3. Biogenic elemental selenium and tellurium precipitates

Elemental selenium precipitates are often the end result of both detoxification and respiratory selenium reduction mechanisms, and typically form as a crystalline monoclinic or amorphous, red material. Elemental selenium precipitates have been described for a physiologically and phylogenetically diverse range of bacteria including *Shewanella oneidensis*, *Geobacter sulfurreducens*, *Veillonella atypica*, *Bacillus selenitireducens* and *Desulfavibrio desulfuricans* as well as by species such as *Stenotrophomonas maltophilia* and *Sulfurospirillum barnesii* strain SES-3 which have been isolated from known seleniferous environments (71, 82, 121, 124, 155, 164). Selenium oxyanion reduction linked to the formation of an insoluble elemental phase has also been described under aerobic conditions for a number of bacteria including *Pseudomonas stutzeri*, *Bacillus* sp. NS3 and *Stenotrophomonas maltophilia* strain SelTE02 (8, 96, 130).

The exact mechanism and subsequent bacterial localisation of elemental selenium precipitation is the focus of some debate. Bacterially derived Se$^0$ is typically found external to the cell, however intracellular precipitates have been noted within the cytoplasm and the periplasmic space, suggesting that Se
oxyanion reduction may occur through a number of disparate pathways (66, 80, 82, 97, 121, 124, 164).

Gel electrophoresis analysis of selenium-bound proteins by Pearce et al (2009) showed that isolated Se⁰ nanoparticles produced by *G. sulfurreducens* still contained significant concentrations of protein identified as c-type cytochromes, which are abundant on the outer cell membranes of *G. sulfurreducens* and also *S. oneidensis*, and are inferred in transfer of electrons to external solid phase terminal electron acceptors (21, 93, 112). These findings suggest that c-type cytochromes are involved in Se oxyanion reduction, and the findings of Abdelouas et al (1) show isolated *c₃*-cytochromes are solely capable of Se⁶ reduction to Se⁰. It is likely therefore that *G. sulfurreducens* and *S. oneidensis* both utilise cytochromes in the formation of extracellular Se⁰ precipitates.

The extracellular localisation of Se⁰ precipitates for bacteria not known for any mechanism capable of significant electron transfer to the outer cell membrane is therefore troublesome to explain. Intracellular Se⁰ has been identified by Tomei et al (164), Losi and Frankenberger (97) and Kessi et al (80) for *D. desulfuricans*, *Enterobacter cloacae* SLD1a-1 and *Rhodospirillum rubrum*, respectively. Each author postulates a different mechanism of transport for Se⁰ to the extracellular environment; (i) Tomei et al (164) concludes that internally reduced Se⁰ is only released in to the external environment following cell lysis; (ii) Losi and Frankenberger (97) conclude that forming Se⁰ is rapidly removed from within the cell by membrane-associated efflux pumps; and (iii) Kessi et al (80) hypothesise that cytoplasmic Se⁰ precipitates are encapsulated within vesicles which are then able to be excreted from the cell. Oremland et al (121) found that *B. selenitireducens* produced both internal and external Se⁰ precipitates, and deduced that Se oxyanion reduction occurs chiefly at the outer cell surface and is responsible for the majority of Se⁰ precipitates. Internal Se⁰ deposits are likely the result of detoxification mechanisms active within the cells as it is inevitable that some of the soluble Se oxyanions will enter into the cell via sulfate or nitrite uptake mechanisms.
Typically, bacterial precipitation of elemental Se leads to the formation of sub-micron diameter spheres, with the average particle size and size distribution dependent on bacterial species and the conditions under which they were incubated (88, 121, 159). Recent research published by Lee et al (88) and Tam et al (159) saw indications that variations in O₂ and biomass concentrations could lead to decreased particle size and size distributions, respectively, for Se⁰ precipitates produced by *Shewanella sp.* HN-41.

Bacterially precipitated elemental Se has also been reported to be more stable than chemical counterparts. Kessi et al (80), Oremland et al (121) and Pearce et al (124) all report that bacterially synthesised Se⁰ from a diverse range of bacteria remain stable for at least several months, whether remaining in the precipitating solution or transferred into deionised water. Kessi et al (80) also found that abiotically synthesised amorphous red Se⁰ remained stable in used, sterile growth media, however crystallised when suspended in fresh media suggesting that bacterially excreted polymeric substances acted to stabilise the particles, and also found that up to 20mg of protein was associated per millimole of Se. Similar effects have been recorded by Fe nanoparticles bacterially synthesised by *G. sulfurreducens*, where time of flight – secondary ion mass spectrometry was used to identify a surface coating of bacterially originating organic material (24).

Research attempting to fully characterise the effects of proteins on the formation and stability of selenium nanoparticles has recently been undertaken (44, 75, 169). Dobias et al (44) have studied the formation of Se⁰ precipitates by *E. coli* and found four proteins strongly bound to isolated Se⁰ nanoparticles; AdhP, Idh, OmpC and AceA, and note that none of these are associated with Se or Fe metabolism, rather related to energy production or the metabolism of carbohydrate and fatty acids. Dobias et al (44) then continue to explore the relationship between Se⁰ precipitates and protein, finding that decreased size distributions and perfectly spherical morphologies were found with the chemical synthesis of Se⁰. Valueva et al (169) and Johnson et al (75) both explore the relationship of the protein Bovine Serum Albumin (BSA) with Se⁰ nanoparticles. Valueva et al (169) found that stable, spherical amorphous Se⁰
particles were produced abiotically in the presence of BSA, and describe a BSA- Se\(^0\) molecule composed of a Se\(^0\) core surrounded by multiple layers of \(\sim\)1000 molecules of BSA. BSA-stabilised Se\(^0\) produced by Johnson et al (75) was found to display a decreased reactivity owing to a surface passivation of Se\(^0\), however BSA-Se\(^0\) synthesised by Valueva et al (169) was shown to display near identical reactivity in clinical tests looking at the treatment of cancer cells. A second possibility for the origin of the increased stability displayed by these particles is reported by Oremland et al (121), whose findings show that Se\(^0\) precipitated by the Se-respiring bacteria *B. selenitireducens, Sulfurospirillum barnesi* and *Selenihalanaerobacter shriftii* all differ structurally from each other and from chemically synthesised Se\(^0\), either from the reduction of Se\(^{IV}\) by ascorbate or the oxidation of H\(_2\)Se by O\(_2\). They hypothesise that bacterial Se\(^0\) consists of a 3-dimensional ‘net’ of six- and eight-membered Se rings and \(\alpha\)-helical chains, with variations occurring between bacterial species as a result of differences in the enzymes used in the Se reduction pathways. Bacterial precipitation of elemental tellurium has been reported for a wide range of clinically and environmentally isolated bacteria, primarily a result of detoxification mechanisms (119, 161, 183) although bacterial respiratory tellurate/tellurite reduction has been described (10, 30, 167). As with Se, Te oxyanion reduction has been demonstrated for environmental isolates under both aerobic (119) and anaerobic (10) conditions. Te\(^0\) precipitates are typically described as intracellular crystalline deposits, and work by Trutko et al (167) found that localisation of Te\(^0\) crystallites was determined by the location of the active centres of the terminal oxidases in the plasma membrane, with precipitates found on either or both inner and outer membrane surfaces as well as within the periplasmic space. Baesman et al (10) report that *B. selenitireducens* and *S. barnesi* produce both intracellular and extracellular deposits of Te\(^0\), in keeping with the findings of Se\(^0\) precipitates with these microorganisms. Again, it is inferred that extracellular deposits are formed through energy conservation reactions whereas intracellular deposits likely result from detoxification mechanisms.
In contrast to Se\(^0\), bacterial Te\(^0\) precipitates are noted to have similar internal arrangements; trigonal unit cells arranged into an \(\alpha\)-helical structure running parallel to the \(x\)-axis (10). Despite this common internal structure, differences in external structure have been noted when Te\(^0\) is precipitated by a range of bacteria. Baesman et al (10) describe two forms of Te\(^0\) precipitated by \(B.\) selenitireducens and \(S.\) barnesii, the former producing bundles of nanorods whilst the latter produces clusters of spherical nanoparticles.

3.4. **Bacterial formation of selenide and telluride**

Reduction of immobile elemental Se to the mobile Se\(^{II}\) phase by bacteria is rare; however the formation of millimolar concentrations of Se\(^{II}\) has been reported for several species including the environmental isolates \(Geobacter\) sulfurreducens, \(Shewanella\) oneidensis, \(Desulfovibrio\) desulfuricans and \(Bacillus\) selenitireducens as well as the clinical isolate \(Veillonella\) atypica (71, 124, 164). Reduction of Se\(^{IV}\) to Se\(^{II}\) by \(G.\) sulfurreducens and \(S.\) oneidensis is through a monophasic reduction pathway, whereby continual reduction of Se\(^{IV}\) to Se\(^{II}\) occurs via Se\(^0\) (124). For \(B.\) selenitireducens and \(V.\) atypica, the occurrence of Se\(^{II}\) follows the complete removal of Se\(^{IV}\) from solution as Se\(^0\) (71, 124). Reduction of an insoluble Se\(^0\) phase by bacteria represents an issue as to how to transfer respiratory electrons to the external terminal acceptor; for \(G.\) sulfurreducens and \(S.\) oneidensis, the transfer of electrons to external precipitates is well documented via the use of outer membrane c-type cytochromes (21, 93, 112, 124). For \(B.\) selenitireducens and \(V.\) atypica, the mechanism of transfer is unknown however the findings of Pearce et al (124) show that Se\(^{II}\) formation by \(V.\) atypica can be stimulated by the addition of the electron shuttling compound anthraquinone-2,6-disulfonate (AQDS) to aid transfer of electrons to the Se\(^0\) precipitate.

There have been no reports of bacteria that are capable of reducing tellurium to aqueous telluride anions; tellurate and tellurite reduction results in either the formation of elemental tellurium or as a methylated tellurium form.
4 Technological applications of selenium and tellurium

Prior to the discussion of the properties of ‘quantum dots’, it is first necessary to describe the fundamental principles underlying semiconduction and the quantum size-effects of nanomaterials. Fundamental crystalline semiconductor principles and the causes of quantum confinement are described in a number of texts, including “Inorganic chemistry” by Shriver et al (149) and “An introduction to mineral sciences” by Putnis (134), and many review papers including those by Dettmer (37), Trindade et al (166), Murphy and Coffer (110), Murphy (109), Zhang et al (186) and Mansur (100).

4.1 The principles of semiconduction and quantum confinement

In 3-dimensionally ordered crystalline bulk materials, electrons occupy distinct, quantised energy levels (molecular orbitals), which can be considered to be a combination of the individual atomic electron shells. The highest occupied molecular orbital (HOMO) that is occupied at 0\(^\circ\)K is called the valence band, and the next attainable energy level above this is the conduction band (lowest unoccupied molecular orbital; LUMO). Electrons located within the conduction band have sufficient energy to disassociate from a particular atomic orbital and can move freely through the crystal lattice, thus becoming a charge carrier (134, 149). The energy between the valence and conduction bands is variable, dependent on the elemental composition and the atomic structure of the material, and is referred to as the band gap ($E_g$) (110, 166). Located near the valence shell is the Fermi level, an energy band where there is a 50% chance of being occupied by an electron, with a decreasing likelihood extending away from the nucleus. This energy level is also highly variable, dependent on the structure and composition as well as the presence of any defects or impurities (110). Figure 4.1 shows three possible scenarios for the relative locations of the Fermi level, valence and conduction bands; (i) insulating materials, which have large $E_g$ values, typically >4eV. Electrons are unlikely to attain sufficient energy to cross the band gap from the valence band in to the conduction band, and so are highly resistant to electron flow, displaying high resistance. (ii) In conducting
materials (metals), the energy required for an electron to travel through the
crystal lattice is lower than the energy of the HOMO within the valence band,
with the Fermi level existing at some energetically higher point. Therefore even
at 0°K, metals are still capable of transmitting an electrical charge as electrons
can freely pass between atoms.

![Diagram of electron orbitals within a solid material](image)

*Figure 4.1. Electron orbitals within a solid material*

The $E_g$ for conducting materials is typically below $\sim 0.1\text{eV}$, and is easily attainable
for electrons through thermal inputs at room temperature (110, 166). (iii) Intermediate between the properties of insulators, with a high $E_g$, and
conductors, with negligible $E_g$ are semiconductors, with $E_g$ values typically
between $\sim 0.5$ to $\sim 3.5\text{eV}$ (table 4.1) (110). In semiconducting materials, electrons
may obtain sufficient energy to pass from the HOMO to the LUMO via external
stimuli, such as an incident photon, ambient temperature or an electromagnetic
field (figure 4.2) (149).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Band Gap (eV)</th>
<th>Structure</th>
<th>Lattice Spacing (Å)</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicon (Si)</td>
<td>1.11</td>
<td>Diamond (Face centred cubic)</td>
<td>A: 5.43</td>
<td>Integrated circuits</td>
</tr>
<tr>
<td>Cadmium sulphide (CdS)</td>
<td>2.53</td>
<td>Wurtzite (hexagonal)</td>
<td>A: 4.136</td>
<td>Photovoltaics</td>
</tr>
<tr>
<td>Cadmium selenide (CdSe)</td>
<td>1.74</td>
<td>Wurtzite (hexagonal)</td>
<td>A: 4.299</td>
<td>Photovoltaics</td>
</tr>
<tr>
<td>Cadmium telluride (CdTe)</td>
<td>1.50</td>
<td>Zinc blende (cubic)</td>
<td>A: 6.477</td>
<td>Photovoltaic cells, electrooptic modulators</td>
</tr>
<tr>
<td>Zinc selenide (ZnSe)</td>
<td>2.58</td>
<td>Zinc blende (cubic)</td>
<td>A: 5.667</td>
<td>Infrared windows, LEDs</td>
</tr>
<tr>
<td>Zinc telluride (ZnTe)</td>
<td>2.28</td>
<td>Zinc blende (cubic)</td>
<td>A: 6.101</td>
<td>LEDs, solar cells, laser diodes</td>
</tr>
</tbody>
</table>

Table 4.1 Examples of semiconductors with energy band gaps, structures and lattice spacing listed along with some typical uses (After Trindade et al 166).
Figure 4.2. The generation of a photon with a wavelength in the visible spectrum by a CdSe quantum dot. (A) The excitation of an electron from quantised molecular orbitals in the valence band of a quantum dot to the conduction band. (B) The electron in the conduction band and the hole created in the valence band can both be considered charge carriers, and are together known as an exciton with a typical separation of ~1-10nm in semiconducting materials. (C) The relaxation of an electron from the conduction band, and the consequent emission of a photon. After Shriver (149).

Excitation of an electron in to the conduction band of a semiconductor such as cadmium selenide can occur with the absorption of a photon of UV light, as seen in figure 4.2a. This electron is then free to participate in electrical conduction, leaving behind a hole in the valence band. The hole behaves within the lattice as a positive charge carrier with an associated effective mass (110, 166), and the physical separation between an electron and the hole generated (together an exciton, figure 4.2b) can be calculated using equation 1.4.1. Recombination of the exciton involves a decrease in energy from the electron, which is reemitted as a photon, in the case of cadmium selenide, within the visible spectrum at a wavelength of ~720nm (figure 4.2c) (110).
\[ r = \frac{\varepsilon h^2}{\pi m_r e^2} \]

_Equation 1.4.1_. Modified Bohr model equation for the approximation of physical separation of an electron and hole pair, the exciton-Bohr radius, where \( r \) is the radius of the separation, \( \varepsilon \) is the dielectric constant of the semiconductor, \( h \) is Planck’s constant, \( m_r \) is the effective mass of the exciton (electron and hole) and \( e \) is the elementary charge of the electron. After Murphy and Coffer (110) and Trindade _et al_ (166).

Typical values for the mass of the exciton have been empirically determined in the region of 0.1 - 3 \( m_r \) for semiconducting materials, with a calculated exciton-Bohr radius of \( \sim 1 \text{ – 10nm} \) (110, 166). For particles with dimensions that fall below the exciton-Bohr radius, the separation of the electron and hole are restricted by the physical dimensions of the crystal, resulting in an increase in kinetic energy of the exciton owing to increased Coulombic interaction (166). Accordingly, it has been observed that the optical emission spectra of semiconducting nanoparticles display a ‘blue shift’ (a decrease in peak emission wavelength) with decreasing particle size below the exciton-Bohr radius, defining an upper limit to the onset of quantum confinement (figure 4.3) (37, 110, 166). The increase in band gap energy, inversely proportional to the particle radius, can be approximately calculated using equation 1.4.2.

\[
E_{QD} = E_G (\text{bulk}) + \left( \frac{\hbar^2}{8 R^2} \right) \left( \frac{1}{m_e} + \frac{1}{m_h} \right) - \frac{1.8 e^2}{4 \pi \varepsilon_0 \varepsilon R}
\]

_Equation 1.4.2_. Where \( E_{QD} \) is the band gap energy of a quantum dot of a radius \( R \), \( E_G \) is the bulk material band gap, \( h \) is Planck’s constant, \( m_e \) is the effective mass of the electron in the solid, \( m_h \) is the effective hole mass in the solid, \( e \) is the charge of the electron, \( \varepsilon \) is the dielectric constant of the solid, \( \varepsilon_0 \) is the permittivity of a vacuum. (After 110).
Another consequence of decreasing the particle radius below the exciton-Bohr radius is that the energy bands within which the electrons and holes reside can no longer be considered continuous, instead forming discrete energy levels (figure 4.2) and can be considered to be in a transitional state between bulk and elementary materials (37, 109, 110, 166).

![Image](image_url)

*Figure 4.3. Glutathione-stabilised cadmium selenide quantum dots displaying varying colour with particle size.*

4.2 **Zero-dimensional semiconducting ‘quantum dots’**

Owing to the effects of quantum confinement, resulting in a decrease in photoemission wavelength with decreasing particle size, it is possible to use a single excitation source to produce photoemissions ranging from UV to infrared, inclusive of the visible spectrum (37, 100, 109, 110, 166). This raises the possibility of ‘tuning’ a quantum dot for particular applications. Further experimentation has found that in addition to the particle size, the crystal structure and chemical composition of quantum dots can be used to vary photoemission wavelength; e.g. the frequency of photons emitted from the solid solution series of GaP$_x$As$_{1-x}$ varies linearly with $x$ (109).
For this reason, many novel applications are currently being developed to take advantage of this technology. One area which has seen particularly intense research is the field of ‘bioimaging’. Quantum dots have many distinct advantages over traditional organic fluorophores; (i) decreased photobleaching over time, (ii) functionalised quantum dots of varying sizes bound to different areas within a cell will fluoresce different colours under a single excitation source, (iii) they display narrow emission wavelengths, unlike organic fluorophores which may overlap, limiting the number of different dyes used in a single application, (iv) heavy metal quantum dots can easily be viewed under transmission electron microscopes (11, 65, 79, 100, 108, 138, 145, 184, 186). A particularly successful application of quantum dots as fluorescent biomarkers is in the detection of tumours. Bioconjugation of quantum dots with biochemical markers or antibodies specific for cancer cells has been shown effective in identifying tumours, especially coupled with quantum dots that emit in the infrared region of the spectrum, owing to the low absorption by body tissue at these wavelengths (62, 63, 145, 173, 180, 184, 186). Future research with conjugated biochemical markers and quantum dots is aimed at in vivo bioimaging of human cancer tissues, along with possible exploitation of this technology for highly-focused drug delivery, where the release of drugs occurs with the excitation of quantum dots, and cancer treatment through reactive oxygen species generation with absorption of incident IR photons (186).

Chemical applications of quantum dots have been studied for some time, with the photoredox and photocatalytic properties differing between nanoparticles and bulk semiconductors due to the increased bandgaps seen in quantum dots, and therefore increased redox potentials, due to the effects of quantum confinement (114, 166). Henglein (70) and Nedeljkovic et al (114) noted that CdS and CdSe nanoparticles, respectively, were capable of the reduction of CO₂ to formic acid with UV illumination, and the evolution of H₂ was noted for PbSe and HgSe nanoparticles in aqueous solution with UV illumination, reactions which are not seen with bulk materials. The oxidation of alcohols and aqueous sulfite oxyanions by ZnS and CdS nanoparticles with UV illumination have also been reported (70).
A range of technological applications have been envisaged for quantum dots owing to the unique photovoltaic and photoluminescent properties due to quantum confinement. A number of potential technological applications are listed for a range of semiconductor nanoparticles by Trindade et al (166) including as light emitting diodes (25, 85, 141) and the photovoltaic components of solar cells (110, 166).

All of the above applications require specific chemical and physical properties from quantum dots, and it is therefore essential to develop synthesis techniques that can repeatedly and economically produce well crystalline, highly fluorescent semiconductor nanomaterials with a predetermined average particle diameter and narrow size distribution.

One technique that has been extensively developed is the organometallic ‘hot-injection’ synthesis of II-VI semiconductor nanomaterials proposed by Murray et al (111), typically creating CdS, CdSe and CdTe quantum dots. The reactants, dimethylcadmium and tri-n-octylphosphine selenide (TOPSe), are injected into tri-n-octylphosphine oxide (TOPO) heated to ~300°C. The injection of the cold reactants into the hot solvent leads to a rapid cessation in nucleation and growth of the nanoparticles, due to the depletion of the initial reactants and the decrease of the solution temperature to ~180°C (111). The solution can then be slowly heated to allow for the growth of the nanoparticles to the desired size. This technique has several distinct advantages; the development of well crystalline, defect-free quantum dots with a narrow size distribution and high quantum yield, owing to the ability to separate the nucleation and growth phases and the presence of TOPO, which acts as a surfactant limiting particle size, increasing particle stability and inhibiting agglomeration (16, 45, 166).

There are, however, several limitations to organometallic synthesis; the requirement for high temperatures in addition to the use of extremely dangerous reactants (especially organometal compounds such as dimethylcadmium) and a high cost of reactants which may be many times greater than those required for other synthesis techniques (16, 45, 64, 125). Alternative reactants and solvents have been investigated, including the use of organic acid complexed cadmium oxide or cadmium acetate as a replacement.
for dimethylcadmium, elemental chalcogenide phases and a range of organic solvents (45, 126, 135).

Another complication of organometallic synthesis is the necessity to resuspended quantum dots into an aqueous solution for biological applications, requiring the exchange of hydrophobic surfactant molecules for hydrophilic counterparts (16, 45, 125). An alternative technique has been developed for the synthesis of quantum dots in aqueous solution, circumventing the requirement for surfactant exchange. These techniques employ water-soluble reactants, and have been utilised to generate a range of nanomaterials including CdS (172), CdSe (16, 143), CdTe (64, 142), ZnSe (188), and PbS (107).

Typically, H₂Se or H₂Te gas is generated by the dropwise addition of H₂SO₄ to Al₂Se₃ or Al₂Te₃, respectively, with the products removed by continuous flushing with N₂ (16, 64, 143). The carrier gas can then be titrated against NaOH to form NaHSe/NaHTe (36, 143), or pumped directly into the reaction vessel (64, 187, 188) which contains the soluble cation precursor, commonly Cd(ClO₄)₂ (16, 64, 143, 172) or CdCl₂/ZnCl₂ (36, 187, 188). The reaction vessel is then heated under reflux at ~100°C until the desired particle size and photoluminescence is obtained (16, 64, 143, 172). There are numerous advantages to this single-step aqueous synthesis method, including a high reproducibility and ease of scalability that are yet to be matched by organometallic synthesis routes (64). Water soluble reactants have also shown to be more economically viable in the production of large quantities of nanomaterials and the reactants themselves tend to be less toxic (32, 64, 125). It is also shown that it is possible to form smaller quantum dots with the aqueous synthesis method (64, 143). There are also several disadvantages to the formation of quantum dots in aqueous solutions; the lower temperatures employed, limited by the boiling point of water, lead to a decrease in particle crystallinity compared to organometallic synthesis, where temperatures up to ~300°C lead to the annealing of particles (45, 64). Particle size distributions also tend to be broader with aqueous synthesis owing to reduced control of the nucleation and growth stages; however this is overcome by the use of post-preparative size selection methods utilizing non-solvents (64).
Developments in the aqueous synthesis of quantum dots has lead to the use of ‘capping’ agents, surfactants that aim to hinder particle agglomeration and reduce the effect of Ostwald ripening by stabilising nm-scale particles that would otherwise dissolve into solution owing to thermodynamic constraints, decreasing the attainable effective particle size. A number of capping agents have been investigated, including phosphates (153), and a range of thiol-bearing compounds including β-mercaptoethanol, 1-thioglycerol, thioglycolic acid and L-cysteine (64). The high affinity of the thiol-compounds with chalcogenide bearing nanomaterials creates a metal chalcogenide – metal surfactant complex surrounding the forming nanoparticles (61), which has been seen to increase fluorescent efficiency and increase the stability of the nanoparticles by decreasing photo-induced surface oxidation and limiting contact between the nanoparticle surface and the oxidative aqueous environment (45, 61, 64). By altering the thiol capping agent used and the reactant concentrations relative to the thiol compound, it is possible to create quantum dots of a predetermined size, with a specific water solubility and zeta potential (64).

Investigations by Gaponik et al (64) found that refluxing newly formed quantum dots lead to the partial hydrolysis of thiol-bearing compounds, resulting in the incorporation of sulphur into the crystal structure, with a likely increase in sulphur content towards the particles outer edges. In that research, this lead to the formation of an alloyed CdTe$_{1-x}$S$_x$ phase which displayed increased stability and fluorescent efficiency, effectively creating a single-step preparatory technique for core-shell metal-chalcogenide nanoparticles.

A number of sulphurous biological proteins have also been investigated as capping agents, notably bovine serum albumin (BSA) (113, 175) and reduced form of the tripeptide glutathione (GSH) (16, 34, 125, 188). As with the chemical capping agents, the biological components bind to the metal (e.g. cadmium) precursor, and act as templates, hindering particle growth whilst increasing small particle stability (16, 113, 125, 175, 188).

These biological compounds have also been implicated in the in vivo formation of metal chalcogenide nanoparticles formed by a range of microorganisms, often formed as a result of heavy metal detoxification mechanisms (see table.
4.2). Dameron et al (33) found that the yeasts *Candida glabrata* and *Schizosaccharomyces pombe* were capable of producing cadmium sulphide quantum dots following incubation with cadmium sulphate (33-35). Research stimulated from these findings by Mehra et al (104) and Reese et al (136) isolated a number of glutathione-related sulphur-bearing proteins, and deduced that these proteins were acting to sequester Cd$^{2+}$ in a similar manner to the detoxification of Cu$^{2+}$. In addition to this, Cd$^{2+}$ was seen to enhance the biological reduction of sulphur, resulting in the formation of stable CdS nanocrystals capped in the sulphurous proteins, thus reducing bioavailability and toxicity to the yeast cells (33-35, 104, 136).

The findings of Dameron et al (33-35), Reese et al (136) and Mehra et al (104) have recently been reiterated from a materials science viewpoint by Bao et al (12), in which fluorescent CdTe nanoparticles are produced utilising this same Cd$^{2+}$ detoxification mechanism and the *in situ* chemical reduction of the Te$^{IV}$ oxyanion using sodium borohydride, and indicating that microbial metal detoxification mechanisms represent a novel approach to the formation of functional nanomaterials.

Bacterial formation of metal chalcogenide nanoparticles has been reported for a number of bacterial species and chemical compositions (Table 4.2). In most cases, the formation of metal sulphide quantum dots occurs as a detoxification mechanism, similar to that reported for fungal organisms, utilising sulfur bearing protein complexes to bind metal ions prior to immobilisation with sulfur (152, 158). Coupling this detoxification mechanism with chalcogens other than sulfur typically requires an external reducing agent such as sodium borohydride (13), however a number of bacterial species have been shown capable of the reduction of elemental selenium to selenide (124), and the application of these microorganisms to the formation of metal selenide quantum dots has been demonstrated (125).

Ultimately, the application of bacteria in the formation of technologically, industrially and chemically relevant bionanomaterials relies heavily on a microorganism’s response to the chemical precursors and products formed, whether respiratory or detoxification mechanisms are used.
<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Microorganism</th>
<th>Product</th>
<th>Metal</th>
<th>Reactant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungi</td>
<td><em>Fusarium oxysporum</em></td>
<td>CdSe</td>
<td>CdCl₂</td>
<td></td>
<td>Kumar et al</td>
</tr>
<tr>
<td></td>
<td><em>Candida glabrata</em></td>
<td>CdS</td>
<td>CdSO₄</td>
<td></td>
<td>Dameron et al</td>
</tr>
<tr>
<td></td>
<td><em>Schizosaccharomyces pombe</em></td>
<td>CdS</td>
<td>CdSO₄</td>
<td></td>
<td>Dameron et al, Kowshik et al</td>
</tr>
<tr>
<td></td>
<td><em>Tolulopsis sp.</em></td>
<td>PbS</td>
<td>Pb(NO₃)₂</td>
<td></td>
<td>Kowshik et al</td>
</tr>
<tr>
<td></td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>CdTe</td>
<td>CdCl₂</td>
<td></td>
<td>Bao et al</td>
</tr>
<tr>
<td>Bacteria</td>
<td><em>Escherichia coli</em></td>
<td>CdS</td>
<td>CdCl₂</td>
<td></td>
<td>Sweeney et al</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
<td>CdTe</td>
<td>CdCl₂</td>
<td></td>
<td>Bao et al</td>
</tr>
<tr>
<td></td>
<td><em>Klebsiella pneumoniae</em></td>
<td>CdS</td>
<td>Cd(NO₃)₂</td>
<td></td>
<td>Smith et al</td>
</tr>
<tr>
<td></td>
<td><em>Rhodobacter sphaeroides</em></td>
<td>ZnS</td>
<td>ZnSO₄</td>
<td></td>
<td>Bai et al</td>
</tr>
<tr>
<td></td>
<td><em>Veillonella atypica</em></td>
<td>CdSe</td>
<td>Cd(ClO₄)₂</td>
<td></td>
<td>Pearce et al</td>
</tr>
</tbody>
</table>

**Table 4.2.** A range of fungi and bacteria have been used to form metal chalcogenide bionanomaterials from a number of metal precursors.

An alternative to the lengthy and costly process of bioengineering microorganisms for desired characteristics is to search the natural environment where desired properties would be displayed as a consequence of geochemical conditions. Investigations in to these environments may then yield bacterial
species that can be employed in a ‘greener’, more environmentally-friendly and more economically viable nanomaterial synthesis pathway.

References


76. **Jones, J. B., G. L. Dilworth, and T. C. Stadtman.** 1979. Occurrence of Selenocysteine in the Selenium-Dependent Formate Dehydrogenase of


174.


171. **Vlassov, K. A.** 1966. Geochemistry and Mineralogy of Rare Elements and Genetic Types of Their Deposits. Israel Program for Scientific Translations (Originally in Russian, Published by Academy of Sciences of the USSR, State Geological Comittee of the USSR).


Nanoparticles formed from microbial oxyanion reduction of toxic group 15 and group 16 metalloids

INTRODUCTION

Environmental Significance of Group 15 and 16 Toxic Metalloids

Selenium, tellurium, and arsenic are present naturally in aquatic and terrestrial environments and share many similar biogeochemical characteristics. These elements are released into the environment through the weathering and decomposition of minerals contained within a variety of lithologies, with slow release rates resulting in low environmental concentrations. Selenium, tellurium, and arsenic occur in several oxidation states as oxyanions (e.g., selenate \( \text{SeO}_4^{2-} \), selenite \( \text{SeO}_3^{2-} \), tellurate \( \text{TeO}_4^{2-} \), tellurite \( \text{TeO}_3^{2-} \), arsenate \( \text{HAsO}_4^{2-} \), and arsenite \( \text{HAsO}_3^{2-} \)) in their native elemental states [e.g., Se(0), Te(0)] or in their most reduced states as selenide (-II) and telluride (-II) or arsenide/arsines (-III). These elements can be methylated through microbial activity to form compounds such as dimethylselenide (Ehrlich, 2002; Masscheleyn, et al., 1990), dimethyltelluride (Basnayake et al., 2001; Fleming and Alexander, 1972), and methylarsonous acid (Dopp et al., 2004) as well as a variety of toxic methylated arsine gases (Yuan et al., 2008). These elements are also found as analogues of sulfurous proteins such as selenocysteine and selenomethionine (Bock et al., 1991; Jones et al., 1979; Stolz et al., 2006; Zannoni et al., 2008), tellurocysteine, telluromethionine (Zannoni et al., 2008), and the arsenic-containing amino acid, arsenomethionine (Dembitsky and Levitsky, 2004).

In aerobic environments, these three elements all occur as readily soluble oxyanions. In anoxic environments, Se and Te are predominantly present in their (IV) oxidation states or as their insoluble elemental forms [i.e., Se(0), Te(0)] or arsenic (III) state, as \( \text{H}_3\text{AsO}_3 \) or \( \text{H}_2\text{AsO}_4^- \) (Stolz et al., 2006), which can also form thioarsenate/thioarsenite complexes in lieu of the oxyanions when there is reactive sulfide present (Planer-Friedrich et al., 2006, 2009). Despite their low crustal abundances, the potential toxic and teratogenic effects of these elements are of major concern and, in aquatic environments, especially those under certain evapoconcentrative conditions, they can attain relatively high concentrations.
In such locales, they can accumulate to micromolar (Oremland et al., 1989, 2000) or even millimolar levels (Oremland et al., 2005). During recent decades, anthropogenic activities such as mining, irrigated agriculture, petrochemical refining, and industrial manufacturing operations have exacerbated the problems associated with these elements in the environment (Lemly, 2004). This has resulted in several high-profile pollution incidents, including wildfowl deaths at the Kesterson reservoir (California) due to selenium contamination (Presser, 1994), and human arsenicosis as a result of arsenic contamination in drinking water wells in Bangladesh and West Bengal (e.g., Ahmed et al. [2006]). Consequently, there has been substantial interest in the cycling of these elements in the environment, particularly with respect to changes in speciation as a result of microbial activity.

A surprisingly wide range of environmentally and clinically isolated microbes are capable of altering the chemical state of Se, Te, and As by a variety of oxidation, reduction, or methylation reactions. These are achieved for purposes of respiration, detoxification, and the maintenance of redox poise and, in some cases, to serve as inorganic electron donors for chemo- and photoautotrophic growth (Table 1). Thus, microbes play an important role in the cycling of these elements between reservoirs in the environment (Oremland et al., 2004; Shrift, 1964). The full redox cycle of Se speciation observed in nature is influenced by microbial activity, with microbes controlling both the oxidation and reduction of Se (Dowdle and Oremland, 1998). To date, no environmental cycle has been developed for Te, although it has been reported for nearly a century that microbes can reduce tellurite to elemental tellurium (Klett, 1900) and can methylate tellurium oxyanions (Basnayake et al., 2001; Fleming and Alexander, 1972). The activities of As-metabolizing microbes can affect the speciation and mobility of As in the environment, through arsenate respiration and nitrate-linked anaerobic oxidation of arsenite.

The toxicity of the metalloids Se, Te, and As is due to the disruption of thiol intracellular biochemistry through the formation of stable, long-lived sulfur complexes (Zannoni et al., 2008) and, in the case of As, by substitution for phosphorus, thereby disrupting cellular metabolism. However, both Se and As are readily assimilated by microbes, and Se is an essential trace element present in naturally occurring proteins such as selenocysteine (Bock et al., 1991; Stolz et al., 2006; Zannoni et al., 2008). No biochemical use for Te has been as yet described and it is significantly more toxic than Se, disrupting metabolic processes through its strong oxidizing potential (Salminen et al., 2006). Nonetheless, microorganisms have evolved a variety of resistance mechanisms to the presence of Te-oxyanions (Chasteen et al., 2009).

Microbial resistance to these three toxic elements has been well-documented and relies upon a number of different strategies, depending upon the element in question and its chemical state. The basis of the resistance to \( \text{As} (\text{V}) \) is the reductive expulsion from the cytoplasm as \( \text{As} (\text{III}) \) (Bhattacharjee and Rosen, 2007), while for Se and Te the reduction of cytoplasmic oxyanions to their insoluble and nontoxic elemental states \([\text{Se} (0), \text{Te} (0)]\) establishes resistance. In some microorganisms this results in the accumulation of external bio-minerals associated with the outer cell envelope. Due to a range of biotic and abiotic factors associated with the templating environment in which these bio-minerals are formed, the bio-mineral phases are often nanoscale in dimension. Selenium, tellurium, and arsenic have optoelectrical properties, that is, they have the potential to convert light energy into electricity (and vice versa); thus, these bionanominerals have possible applications in novel photonic devices (Stolz and Oremland, 1999). The production of such desirable nanoscale materials using a biosynthetic route, thereby eliminating toxic organic solvents and minimizing expensive high-temperature processing, is, as yet, a largely unexplored and unexploited area of considerable potential. Indeed, the possibility
### TABLE 1  Some examples of microbial interaction with Se, Te, and As

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Environment</th>
<th>Starting material</th>
<th>End product</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pterotrigonia brevicula</td>
<td>Mine water, PA, USA</td>
<td>Te IV</td>
<td>Te⁰, (CH₃)₂Te gas</td>
<td>Smithers and Krouse, 1967</td>
</tr>
<tr>
<td>Acidithiobacillus ferrooxidans</td>
<td>Clinical isolate, human skin</td>
<td>Se⁻²</td>
<td>Se⁰</td>
<td>Torma and Habashi, 1972</td>
</tr>
<tr>
<td>Corynebacterium</td>
<td>San Francisco Bay mud flat, CA, USA</td>
<td>Se⁰, Se⁴, Se⁶, org-Se</td>
<td>Se⁰ (volatile alkylselenides)</td>
<td>Doran and Alexander, 1977</td>
</tr>
<tr>
<td>Methanococcus vannili</td>
<td>San Francisco Bay mud flat, CA, USA</td>
<td>Se⁴</td>
<td>Se⁰ (organic-Se)</td>
<td>Jones et al., 1979</td>
</tr>
<tr>
<td>Desulfobulbus desulfuricans</td>
<td>Drainage water</td>
<td>Se⁶, Se⁴ Te⁴</td>
<td>Aqueous HSe⁻², Te⁰</td>
<td>Lloyd et al., 2001; Zehr and Oremland, 1987</td>
</tr>
<tr>
<td>Pseudomonas stutzeri</td>
<td>Termite gut and bovine men fluid, respectively</td>
<td>Se⁴</td>
<td>Se⁰</td>
<td>Lottie et al., 1992</td>
</tr>
<tr>
<td>Citrobacter, Wolinella succinogenes</td>
<td>Upper Mystic Lake, MA, USA</td>
<td>Se⁴, Se⁶, As⁵ and S₂O₃⁻</td>
<td>Se⁰, As⁰, AsS-like mineral</td>
<td>Herbel et al., 2002; Tomei et al., 1992</td>
</tr>
<tr>
<td>Thauera selenatis</td>
<td>San Joaquin Valley, CA, USA</td>
<td>Se⁴</td>
<td>Se⁰ (in presence of nitrate)</td>
<td>Macy et al., 1993</td>
</tr>
<tr>
<td>Chrysiogenes arsenatis</td>
<td>Gold mine wastewater</td>
<td>As⁵</td>
<td>As⁰</td>
<td>Macy et al., 1996</td>
</tr>
<tr>
<td>Desulfotomaculum auripigmentum</td>
<td>Upper Mystic Lake, MA, USA</td>
<td>As⁵</td>
<td>As⁰</td>
<td>Newman et al., 1997</td>
</tr>
<tr>
<td>Enterobacter decae SLD1a-1</td>
<td>San Joaquin Valley, CA, USA</td>
<td>Se⁶, Se⁴</td>
<td>Se⁰ nanospheres</td>
<td>Losi and Frankenberger, 1997</td>
</tr>
<tr>
<td>Rhodobacter sphaeroides</td>
<td>Pond water, Hyderabad, India</td>
<td>Se⁶, Se⁴</td>
<td>Se⁰ (organic Se and alkylselenides), Se⁰ (red amorphous and black vitreous)</td>
<td>Van Fleet-Stalder et al., 2000; Yamada et al., 1997</td>
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<tr>
<td>Bacillus selenitireducens</td>
<td>Mono Lake, CA, USA</td>
<td>Se⁴, Te⁴, As⁵</td>
<td>Se⁰ nanospheres, Te⁴ nanorods/rosettes</td>
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<tr>
<td>Bacillus arseniigenes</td>
<td>Mono Lake, CA, USA</td>
<td>Se⁰, As⁵</td>
<td>Se⁴</td>
<td>Blum et al., 1998</td>
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(Continued)
### TABLE 1  Some examples of microbial interaction with Se, Te, and As—Continued

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<th>Microbe</th>
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<td>Marine sediment, Sapelo Island, GA, USA</td>
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<td><strong>Leptothrix MnB1</strong></td>
<td>. . . Tin of milk with a fishy odor</td>
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<td>Knight and Blakemore, 1998</td>
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<td>Aberjona watershed, MA, USA</td>
<td>As(^\circ)</td>
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<td><strong>Geobacillus stearothermophilus V</strong></td>
<td>Massie Slough, NV, USA</td>
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<td>Se(^{\text{II}}), Se(^{\text{IV}}) (nanospheres)</td>
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<td><strong>Sulfurospirillum barnesi</strong></td>
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<td>Te(^{\text{II}}) granules, (CH(_3))(_2)Te gas</td>
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<td>K-27 K-esterson Reservoir, CA, USA</td>
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<td><strong>Pyrobaculum aerophilum</strong></td>
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<td><strong>Pseudomonas fluorescens</strong></td>
<td>Dead Sea sediments, Israel</td>
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<td>Se(^{\text{II}}) (vola- tile alkylselenides)</td>
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<td><strong>Sulfurospirillum barnesi</strong></td>
<td>Hot spring, Naples, Italy</td>
<td>Se(^{\text{IV}},\text{Te IV}})</td>
<td>Se(^{\text{II}}), Se(^{\text{IV}}) (nanospheres)</td>
<td>Blum et al., 2001</td>
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<tr>
<td><strong>Pseudomonas aeruginosa, Agrobacterium tumefaciens, Escherichia coli</strong></td>
<td>Dead Sea sediments, Israel</td>
<td>Se(^{\text{IV}}), Se(^{\text{II}})</td>
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<td><strong>Stenotrophomonas maltophilia</strong></td>
<td>Agricultural evaporation pond, Tulare Lake, CA, USA</td>
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<td><strong>Desulfonatronium sp.</strong></td>
<td>Sulfate-reducing biofilm</td>
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<td>Organism</td>
<td>Location/Environment</td>
<td>Species Information</td>
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<td><em>Desulfobulbacea</em></td>
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<td><em>Shewanella</em> sp. strains ER-Se-17L, ER-Te-48, and ER-V-6</td>
<td>Hydrothermal vent fields, Eastern Pacific Ocean</td>
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<td><em>Bacillus selenatetanalis</em></td>
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<td><em>Clostridiaceae</em></td>
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<td><em>Dechloromonas</em> sp.</td>
<td>Rifle, CO, USA</td>
<td>Se(^{VI}), Te(^{IV}), Te(^{VI})</td>
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<td><em>Ectothiorhodospira</em></td>
<td>Mono Lake, CA, USA</td>
<td>As(^{III})</td>
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<td><em>Veillonella atypica</em></td>
<td>Clinical isolate, human tonsils</td>
<td>Se(^{IV}), Te(^{IV})</td>
<td>Pearce et al., 2008; this work</td>
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<td><em>Bacillus beveridgei</em> MLTeJB</td>
<td>Mono Lake, CA, USA</td>
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<td><em>Bacillus</em> sp. strain NS3</td>
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<td><em>Geobacter sulfurreducens</em></td>
<td>Surface sediments, Norman, OK, USA</td>
<td>Se(^{IV}), Te(^{VI})</td>
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of using industrial waste as the starting material for synthesis of microbially generated nanomaterials is of particular interest.

Microbial Interaction with Group 15 and 16 Toxic Metalloids

The reduction of selenium oxyanions as terminal electron acceptors is energetically favorable, with the free energy ($\Delta G_m^{\circ}$) of the reduction of $\text{SeO}_4^{2–}$ and $\text{HSeO}_3^–$ coupled with the oxidation of $\text{H}_2$, respectively, yielding $–15.53$ kcal mol$^{-1}$e$^{-1}$ ($–65.0$ kJ mol$^{-1}$ e$^{-1}$) and $–8.93$ kcal mol$^{-1}$e$^{-1}$ ($–37.4$ kJ mol$^{-1}$ e$^{-1}$) (Newman et al., 1998). Selenium oxyanion reduction occurs in a wide range of microbes, including representatives of the Wolinella, Pseudomonas, Sulfurospirillum, Enterobacter, Thauera, Bacillus, Shewanella, and Citrobacter genera (see also Table 1). The various mechanisms of selenium oxyanion reduction by microbes have been reviewed by Zannoni et al. (2008). There are four modeled pathways through which the reduction of selenium oxyanions may occur: (i) a painter-type reaction in which the selenium oxyanions are reduced to elemental selenium by glutathione via several intermediates such as selenodiglutathione and glutathioselelenol; (ii) oxyanion reduction by cytosolic and periplasmic oxidoreductases; (iii) abiotic reactions with metabolic products, e.g., during the reduction of sulfate to sulfide, the sulfide can react with selenium to produce elemental selenium and sulfur; and (iv) the production of elemental selenium by a reaction with the biologically derived chelating agent pyridine-2,6-bisthiocarboxylic acid (Cortese et al., 2002; Hockin and Gadd, 2003; Stolz and Oremland, 1999). Microbes can also reduce the solid elemental selenium phase to produce volatile alkylselenides, with dimethyl selenide and dimethyl diselenide as the most common forms in which Se is present as Se(-II). Aqueous selenide ($\text{HSe}^–$) can also be a product of Se(0) reduction, which reacts with metal cations to form a solid precipitate, e.g., FeSe. Gaseous $\text{H}_2\text{Se}$ formed by cultured anaerobic bacteria will rapidly auto-oxidize back to Se(0) if exposed to O$_2$, typically caused by changing the incubation headspace from N$_2$ to air. The production of Se(-II) after Se(0) formation can be either biphasic, as with the Se(IV)-respiring gram-positive haloalkaliphile, Bacillus selenitireducens (Herbel et al., 2003) or continuous, as reported for Geobacter sulfurireducens and Shewanella oneidensis (Pearce et al., 2009).

The biochemistry of dissimilatory selenate reduction has been studied most extensively in the Se(VI) respirer, Thauera selenantis (Macy et al., 1993; Rech and Macy, 1992). Selenate reductase from this organism has been purified and characterized (Kraft et al., 2000; Maher et al., 2004; Schröder et al., 1997), and is placed in the class of Mo-containing reductase enzymes (McEwan et al., 2002). A number of microbes can carry out the reduction of Se oxyanions without a direct linkage to energy conservation. Among these Enterobacter cloacae has been best characterized (Leaver et al., 2008; Losi and Frankenberger, 1997) because it has proven amenable to genetic manipulation via knockout mutagenesis (Ma et al., 2007, 2009; Yee and Kobayashi, 2008). E. cloacae is a facultative anaerobe and forms external accumulations of Se nanospheres (Yee et al., 2007) that appear outwardly identical to those produced by fastidious Se-respiring anaerobes (Oremland et al., 2004). This makes E. cloacae a more practical model organism to work with. In contrast, surprisingly little is known as yet about the biochemistry of dissimilatory Se(IV) reduction.

Microbial respiration of tellurate has been claimed to be achieved by an as yet unidentified microbe isolated from hydrothermal vents located in the eastern Pacific Ocean (Csontonyi et al., 2006). Work by Baesman et al. (2007) showed that Sulfurospirillum barnesii and B. selenitireducens were capable of respiratory growth using tellurate and tellurite oxyanions, respectively, which was quantitatively coupled to their oxidation of lactate to acetate, together with CO$_2$. Although both these strains grew well on millimolar levels of selenium oxyanions (as well as arsenate), they both proved sensitive to tellurium oxyanions, with con-
centrations of ≥1 mM completely inhibiting growth. Hence Te-dependent growth had to be achieved by the sustained pulsing of cultures with sublethal quantities (~0.6 mM) of Te(IV) or Te(VI). Most recently, a microbial species was isolated from Mono Lake by using ~10 mM Te(IV) routinely in the enrichment/isolation process. This organism, Bacillus beveridgei, is capable of growth using millimolar concentrations of Te(VI) or Te(IV) (Baesman et al., 2009). Microbial reduction of tellurate and tellurite results in the formation of elemental tellurium or the more reduced methylated form, but, in marked contrast to the case for selenium, not in the formation of aqueous telluride anions. An ability to form biotelluride anions for the production of telluride nanomaterials (e.g., CdTe) would be of great significance because of their use in the development of photonic devices. The fact that this can be achieved for selenium but not for tellurium suggests a possible divergence (or limitation) in the biochemical pathways for dissimilatory Te-oxynion reduction that are thought to be similar to those for selenium. However, two Pseudomonas aeruginosa strains and Rhodobacter sphaeroides (Moore and Kaplan, 1992) have been shown to use c-type cytochromes and cytochrome oxidases during reduction of tellurate, and tellurite reduction by Escherichia coli involves a quinol oxidase system. None of these three organisms is a “true” Te-respirer that can actually conserve energy for growth as do B. sel- enitireducens, B. beveridgei, or S. barnesii. The biochemical pathways for dissimilatory Te-oxynion reduction and how they conform to or diverge from those for Se oxanions is a research question that has yet to be addressed. Indeed, only a few novel microorganisms have been examined to date for their ability to conduct dissimilatory reductions of Te oxanions. Hence, the possibility that some as yet undiscovered microbe exists with the ability to form telluride from more oxidized forms of Te is still a distinct possibility.

There are four basic processes of microbial arsenic transformation: methylation, demethylation, oxidation, and reduction (Stolz et al., 2006). Methylation involves the reduction of arsenate followed by the oxidative addition of a methyl group to form a range of different compounds with As in the V, III or –III state. Little is known about the mechanisms of demethylation, but if the pathway is the reverse of that described for methylation, it would involve reductive elimination and oxidation of the center, restricting demethylation to As(V) species (Stolz et al., 2006). Arsenate reductases and arsenite oxidases have been purified for a limited number of organisms and are members of the dimethyl sulfide reductase family of molybdenum enzymes. Both the respiratory arsenate reductase and arsenite oxidase are heterodimers with similar structures and molecular weights, but are thought to be unidirectional in their mode of action (Silver and Phung, 2005). However, a reverse functionality for the arsenate reductase of Alkalilimno- nicola ehrlichii was discovered (Hoeft et al., 2007; Richey et al., 2009) whereby it oxidized As(III). This mechanism was also found in a photosynthetic bacterium of the Ectothiorhodospira clade of Gammaproteobacteria as the means by which As(III) served as an electron donor for anoxygenic photosynthesis (Kulp et al., 2008). The implications that this discovery has for microbial evolution on Earth have been recently reviewed (Oremland et al., 2009).

Applications of Group 15 and Group 16 Metalloid Bionanoparticles

Microbial interaction with Se, Te, and As results in the production of nanoparticles (>100 nm) with unique physical, optical, and electrical properties that are not representative of their bulk equivalents as a result of (i) the ratio of surface area to volume and (ii) unique surface properties in particles <10 nm, and (iii) the onset of quantum confinement where the particle’s band gap is size dependent. Of particular interest are the optoelectronic properties exhibited by nanominerals composed of...
Group 15 and 16 elements, which result in the conversion of incident light energy into electricity and vice versa (Baesman et al., 2009). Microbially produced nano-sized materials comprising elemental Se, Te, and As, as well as those in combination with metallic/nonmetallic elements (e.g., CdSe, As$_2$S$_3$), have a range of technological, medical, and environmental applications. The process of microbial nanomineral formation itself can be used to efficiently remove Se, Te, and As oxyanions from drinking water, groundwater, or wastewater. This type of anaerobic process offers advantages over aerobic processes in terms of low sludge production, smaller reactor volumes, and cost savings in aeration and nutrient supply (Lenz et al., 2008). Nanoparticles of elemental Se can be used in a photocatalytic process to reduce environmentally harmful organic dye contaminants, as shown by the reduction of methylene blue (Nath et al., 2004). Se(0) nanospheres have also been shown to be the cheapest and most efficient sorbents of mercury vapor with potential applications in the safe disposal of compact fluorescent lamps (Johnson et al., 2008).

Technological applications of Se(0) and Te(0) nanoparticles include their use in photocopiers, microelectronic circuits, and solar cells as a result of their photo-optical and semiconducting physical properties (Baesman et al., 2007; Oremland et al., 2004). The fluorescence absorption and emission of CdSe and ZnSe quantum dots are conveniently tunable by their size, resulting in a range of applications including optoelectronic devices, light sensors, and high-purity emission lasers (Dettmer, 1988; Pickett and O’Brien, 2001) and as powerful probes for the labeling of biological components (Giepmans et al., 2006). CdSe nanoparticles have also been shown to act as very efficient and highly selective catalysts for the reduction of aromatic azides to aromatic amines when activated by light (Warrier et al., 2004). Pearce et al. (2008) have shown that nanoscale, luminescent CdSe/ZnSe can be produced by Veillonella atypica using an environmentally friendly, aqueous-based synthesis route. Polycrystalline phases of the chalcogenide minerals realgar (As$_2$S$_3$) and duranusite (As$_4$S$_9$), produced by the Shewanella sp. strain HN-41 in the form of nanotubes, behave as metals and semiconductors in terms of their electrical and photoconductive properties, respectively, and have potential applications in nano- and optoelectronic devices (Lee et al., 2007).

With regard to medical applications, much recent attention has been given to the antioxidant properties of Se(0) nanospheres, which have the capacity to limit the damage caused by free radicals and reactive oxygen species (Gao et al., 2002). People from many parts of the world do not consume enough selenium in their diets, and several recent studies have suggested a link between cancer and selenium deficiency. Nanoparticulate Se(0) is the least toxic of all Se supplements and is effective as a chemopreventive agent (Peng et al., 2007).

The process of microbial nanoparticle formation may also result in the production of novel materials with possible commercial applications that cannot be synthesized by traditional inorganic approaches. Thus, primary research investigations into how these nanoparticles are formed are well justified. Such efforts would include the scrutiny of the proteins, lipids, and polysaccharides that unite cellular processes with inorganic substrates, thereby allowing their initial adhesion to the cell envelope, and subsequent electron transfer and dissimilatory reduction of the metalloids in question. Oremland et al. (2004) showed that microbial Se(0) can have unique structural and spectral features that vary not only from chemically derived materials, but also between bacterial species. Electron microscopy revealed that the Se(0) nanoparticles were encapsulated by a biologically derived exopolymer. Redox-active proteins in the form of $\epsilon$-type cytochromes and ferredoxin were also found to be associated with postreduction mineral phases during the biotransformation of Se oxyanions by G. sulfurreducens and may therefore be involved in the formation of Se(0) nanospheres.
(Pearce et al., 2009). Microbiologically produced Te(0) has distinct characteristics not replicated by chemical methods (Baesman et al., 2007). Fourier transform infrared spectroscopy of Te(0) nanorods produced by *B. selenitireducens* revealed the presence of functional amide groups on the Te(0), suggesting the attachment of cell wall proteins to the Te(0) (Baesman et al., 2007). As-S nanotubes, formed via the reduction of As(V) and S\textsubscript{2}O\textsubscript{3}\textsuperscript{2-} by *Shewanella* sp. strain HN-41 were shown to be associated with EPS-containing polysaccharides (Lee et al., 2007). It is possible that specific proteins serve as templates for assembly/precipitation of Se, Te, and As as nanoparticles and that differences in protein structure could produce the geometric and spectral variations between nanoparticles formed by diverse prokaryotes that interact with oxyanions of these elements. What follows is a brief survey that gives some specific examples of the types and variety of microbially produced Se, Te, and As nanominerals.

**CHARACTERISTICS OF ELEMENTAL Se AND Te BIONANOPARTICLES**

A range of *Bacteria* and *Archaea* can link the oxidation of organic substrates or H\textsubscript{2} to the reduction of selenium oxyanions under a variety of conditions (Table 1). Despite the variety of reduction conditions, the end products of these reactions are predominantly red, amorphous, or monoclinic allotropes of Se(0), in the form of small (~100- to 200-nm) spheres (Fig. 1). The spheres in Fig. 1A were formed under anaerobic conditions at 25°C by the reduction of Na\textsubscript{2}SeO\textsubscript{4} (3 mM) using growing cells of the Mono Lake isolate *B. selenitireducens*, in carbonate-bicarbonate-buffered media (pH 9.8, salinity 56 g/liter), containing lactate as the electron donor, with vitamins and yeast extract added as growth supplements (Oremland et al., 2004). *Bacillus* sp. NS3 cells, isolated from Punjab, India, grown in tryptone soy broth at neutral pH and 30°C, reduced Na\textsubscript{2}SeO\textsubscript{4} (5mM) under anaerobic conditions at pH 7.5 formed the Se(0) nanospheres in Fig. 1C and D, respectively (Pearce et al., 2009). Although the morphologies of the Se(0) nanospheres are similar, Oremland et al. (2004) have shown that the Se molecular chain structural orientation and the optical properties of these materials can be very different, depending on the organism, possibly because of the involvement of different reductase enzymes for the different microbes. The spectral properties of these biogenic Se(0) materials are also substantially different from their chemically formed counterparts (Oremland et al., 2004). There is evidence that morphologies of Se(0) other that the red amorphous nanospheres in Fig. 1A to D can be also be produced by microbial communities that exist in extreme environments, such as the mineral-rich, highly alkaline (pH = 9.8) and hypersaline (salinity = ~75 to 90 g/liter) ecosystem of Mono Lake, California. Sediment (4 ml) collected from the hot spring area of Paoha Island, was added to 16 ml sterile, basal salts medium (Switzer Blum et al., 2009) at pH 9.3 and dispensed into serum bottles (30 ml bottle volume; 20 ml slurry volume) under anaerobic conditions. Sodium selenite (2 mM) was added to all bottles as an electron acceptor, and molecular hydrogen was provided as the electron donor. Killed controls were autoclaved (250 kPa, 121°C, 60 min). The slurries were incubated in the dark at 43°C. The live sediment slurries turned black within 24 h (Fig. 1E, inset), without progressing through an intermediary red phase. No such color change was observed in the killed controls. Scanning electron micrographs (Fig. 1E) show that the Se(0) precipitates formed after incubation of Se(IV)-amended sediment slurries with hydrogen have a plate-like structure and are relatively large (200 to 300 nm) compared with the amorphous red Se(0) nanospheres in Fig. 1A to D. Energy dispersive X-ray spectroscopy (EDS) confirmed that the observed
The microbial diversity of Se-reducing prokaryotes, employing a correspondingly vast range of enzymatic reactions to form Se(0), offers the intriguing potential to tailor the properties of biologically based Se bionanominerals for specific applications in the field of nanotechnology. Relatively facile washing treatments employed to remove the biomass from microbially produced Se(0) nanospheres have also resulted in the production of novel Se(0) phases, including clusters of hexagonal nano-rods, further increasing the “toolbox” of Se(0) nanomaterials that can be made available from inexpensive starting materials and low cost bio-manufacturing procedures (Prakash et al., 2009). Figure 2 shows how Se(0) nanospheres (Fig. 1B) produced by either growing cells of *Bacillus* sp. strain NS3 in tryptone soy broth or by “resting” cells of *Bacillus* sp. strain NS3 in 3-(N-morpholino)propanesulfonic acid buffer...
(20 mmol × l⁻¹, pH 7.5), can be manipulated by postpreparative acetone-washing procedures to form structures with completely different sizes and shapes (Fig. 2A and B).

In contrast to Se-oxyanion reduction, the reduction of Te oxyanions results in the production of Te(0) with a range of basic morphologies including Te rosettes, Te granules, Te nanospheres, and Te nanorods, the latter being the most commonly formed precipitate. Figure 3 shows examples of some of these different morphologies. The medical isolate *V. atypica* cannot use Te oxyanions as terminal electron acceptors for anaerobic respiration. However, this organism is capable of forming ~100-nm Te(0) nanorods, which can be seen protruding from the spherical cells, as well as aggregating into extracellular clusters (Fig. 3A), when grown under anaerobic conditions in the presence of Te(IV) at 37°C in a rich medium containing yeast extract and lactate. The freshwater isolate *S. barnesii* forms both intracellular and extracellular irregularly shaped, ~20-nm-sized nanospheres of crystalline Te(0) that coalesce together to form larger, ~500- to 1,000-nm clusters attached to the cell surface when using pulsed additions of Te(VI) as a terminal electron acceptor for anaerobic respiration, in a defined medium containing lactate at 28°C (Fig. 3B). The Te(0) precipitates produced by the haloalkaliphilic Mono Lake isolates, *B. beveridgei* strain MLTeJB (Fig. 3C) and *B. selenitireducens*, during Te(IV)-dependent growth in a basal salts medium containing yeast extract and lactate at 28°C, are rod shaped but are much larger than those formed by *V. atypica* (Fig. 3A). It is possible that either the differences in Te(0) morphology, shown in Fig. 3, can be explained in terms of rate of reduction (reduction rate for *V. atypica* > *S. barnesii* > *B. selenitireducens*) or the Te(0) morphology may be controlled by different rate-limiting steps along the specific reduction pathways; Te(0) production by *S. barnesii* is a two-step process involving reduction of Te(VI) to Te(IV), followed by reduction of the latter to Te(0). Despite the differences in external morphology, analysis of various Te(0) precipitates using Raman spectroscopy revealed that they have similar internal structures, possibly as a result of the fundamental trigonal alignment features of the Te chains (Baesman et al., 2007). This is in contrast to biogenic Se nanospheres, which have a similar outward appearance but differ with respect to their spectral properties.

Control of biogenic Te(0) precipitate morphology is possible, not only through the application of different Te-oxyanion-reducing organisms, but also via alteration of the growth conditions for a particular organism. Figure 4 shows the Te(0) precipitates formed by the subsurface isolate *G. sulfurreducens*. In an attempt to determine whether *G. sulfurreducens* could couple growth to the reduction of Te(IV), cells were grown in an acetate-amended modified freshwater medium with 1 mM Te(IV) as the only available terminal electron acceptor. The number of cells did not increase over time, indicating that *G. sulfurreducens* was unable to use Te(IV) as a terminal electron acceptor for growth; however, reduction of Te(IV) by the organism was revealed by the formation of a fine, black precipitate (Fig. 4A, inset). The whole mount transmission electron microscopy (TEM) image of the Te(0) precipitates produced by *G. sulfurreducens* under these “growing” conditions (Fig. 4A) shows ~100-nm-sized nanospheres on the cell surface. Confirmation of the Te(0) composition of the nanophase produced was confirmed by EDS (data not shown). High-resolution TEM of these nanospheres (Fig. 4B) reveals that they are composed of much smaller (<10 nm) particles. In contrast, nongrowing or “resting” anaerobic cells of *G. sulfurreducens*, resuspended in tricine buffer and exposed to 1 mM Te (IV), in the presence of anthraquinone disulfonic acid as an electron shuttle with hydrogen as the electron donor at 30°C, produced a dense, black precipitate (Fig. 4C, inset). The scanning electron microscopy (SEM) and high-resolution TEM images of these precipitates in Fig. 4C clearly show an entirely different morphology consisting of ~100-nm-sized nanorods of Te(0). The composition and highly crystalline nature of the Te(0) nanorods
FIGURE 2  Electron micrographs of elemental Se precipitates produced by Bacillus sp. strain NS3. (A) SEM image of solvent-washed Se precipitates produced by “growing” cells. (B) SEM image of solvent-washed Se precipitates produced by “resting” cells and (C) a representative EDX spectrum.
FIGURE 3  Electron micrographs of elemental Te precipitates. (A) SEM images of Te nanorods produced by *V. atypica*. (B) SEM images of Te nanogranules produced by *S. barnesi*. (C) SEM images of Te nanorods produced by *B. beveridgei* strain MLTeJB (inset shows EDX spectrum).
were confirmed by EDS (Fig. 4D) and high-resolution TEM (Fig. 4C) respectively. The presence of sulfur in association with the Te(0) nanorods could result from thiol groups on the surface, indicating a role for the glutathione and thioredoxin redox system in Te(IV) reduction by \textit{G. sulfurreducens} (Turner et al., 2001). Baesman et al. (2007) also noted differences between the Te(0) nanorods formed by cell suspensions of \textit{B. selenitireducens} and those formed during growth experiments. This was attributed to the fact that cell wall proteins remained firmly attached to the Te(0) nanorods produced by the cell suspensions, but this
strong attachment of the Te(0) nanorods to the cell surface was eliminated by the presence of cysteine in the medium for the growing cells. As shown in Table 1, many organisms are capable of reducing both Se(IV) and Te(IV); however, only the *B. beveridgei* strain MLTeJB, along with certain isolates from marine hydrothermal vents (Rathgeber et al., 2002), are capable of reducing significant quantities of both of these oxyanions. During the study of the growth of strain MLTeJB under different electron donor and acceptor conditions, it was found that cells grew on up to 10 mM Te(IV), with excess lactate, and reduced it to Te(0) as the sole reduction product. This result contrasts with growth on Se(IV), where Se(–II) was observed as an end product, as well as Se(0). Subsequent addition of Se(IV) (5 mM) to the reduced suspension of cells and Te(0) resulted in reduction to Se(0) and Se(–II), along with the development of a yellow color in the media over time (Fig. 5C, inset). Figure 5A shows cells of strain MLTeJB, grown in the presence of Te(IV) and Se(IV), encrusted with mixed Se/Te precipitate. The yellow solution was separated from the biomass mass by filtration through a 0.22-µm filter and then centrifuged at 30,000 × g for 5 min to remove any remaining nanoparticles. Oxidation of the yellow solution resulted in the formation of a dark-red precipitate (Fig. 5C inset). SEM images of this precipitate showed that it had nanospherical morphology and was composed of both Se and Te (Fig. 5B and C). These results suggest that, despite the lack of Te(–II) formation during reduction of Te(IV), strain MLTeJB is able to produce a reduced soluble Te phase under certain circumstances, i.e., in the presence of a mixture of Te(0), Se(0), and Se(–II). The mechanism of production of this reduced Se/Te phase is not clear, but it is possible that the addition of Se(IV) to the Te(0) suspension stimulated the strain MLTeJB cells to produce a reducing environment in which the further reduction of Te(0) to Te(–II) was possible.

CHARACTERISTICS OF MSe (*M* = Cd, Zn) BIONANOPARTICLES

For certain organisms, Se(0) is not the end of the reduction pathway for Se oxyanions, and they can mediate the biomethylation and volatilization of Se(0) to form organo-Se

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**FIGURE 5** Electron micrographs of mixed Se/Te precipitate formed by *B. beveridgei* strain MLTeJB grown in the presence of tellurite and selenite (inset shows black precipitate; a color illustration would show a yellow solution). (A) SEM image showing whole cell encrusted with Se/Te precipitate. (B) TEM image showing mixed Se/Te nanosphere precipitates produced upon oxidation of (yellow) solution and (C) accompanying EDS spectrum. (The inset shows Se/Te precipitate, which would be red in a color illustration.)
compounds with the selenium present as Se(–II) (Dungan et al., 2003; Herbel et al., 2003). A limited number of organisms can also extend the reduction pathway beyond Se(0) to form an aqueous selenide end product. Over a period of several hours, V. atypica cells can entirely reduce 5 mM Se(IV) to the soluble Se(–II), present as dissociated HSe– surrounded by water molecules (Pearce et al., 2008). This biogenic Se(–II) solution is produced under ambient conditions, as opposed to the hazardous, expensive production of NaHSe from Al₂Se₃, and can be employed in an aqueous-based, wet chemical synthesis for the fabrication of CdSe/ZnSe quantum dots (Pearce et al., 2008). Precipitation of the biogenic selenide solution with a suitable metal cation, such as ZnCl₂, results in the formation of ~30-nm ZnSe particles. These particles are too large for quantum dot-type applications, because semiconductor quantum dots exhibit tunable optical properties as a result of quantum confinement in the regime below 10 nm (Alivisatos, 1996). However, the employment of simple thiol molecules, such as 2-mercaptoethanol or reduced glutathione, as agents to stabilize the metal cation during synthesis results in the formation of stable metal selenide colloids which, upon heating to 110°C for 2 h, produce quantum-sized (~5 nm), luminescent metal selenide nanoparticles. Color Plate 17A shows the fluorescence spectrum of size-fractionated glutathione-stabilized CdSe quantum dots, illustrating how quantum dots with different emission wavelengths can be formed via a postpreparative size-selective precipitation, involving a nonsolvent such as 2-propanol. Confirmation of the crystallinity and chemical purity of the CdSe quantum dots is provided by the high-resolution TEM image and EDS spectrum in Color Plate 17B.

**CHARACTERISTICS OF AsₓSᵧ BIONANOPARTICLES**

Arsenic sulfides are important infrared transparent materials for a variety of applications such as sensors, waveguides, photonic crystals, and photolithography template materials (Johnson et al., 2005). The fabrication of AsₓSᵧ in the form of nanowires paves the pathway for application in advanced integrated nanophotonic structures and devices. The recent discovery of microbial production of filamentous arsenic-sulfide (As–S) nanowires by Shewanella sp. strain HN–41 (Lee et al., 2007), grown in the presence of As(V) and S₂O₃ under anaerobic conditions, again offers an environmentally friendly alternative to present high-temperature fabrication routes. Figure 6A shows an electron micrograph of the yellow biogenic As–S precipitate (Fig. 6A, inset) produced by strain HN–41, grown in anaerobic basal medium at pH 7.5, supplemented with 20 mM lactate, 10 mM thiosulfate, and 5 mM arsenate at 30°C, revealing that they are long (up to ~30 µm), variable diameter (20 to 100 nm), extracellular As–S filamentous nanotubes. These biogenic As–S precipitates were shown to behave as metals and semiconductors in terms of their electrical properties and were also photoconductive (Lee et al., 2007).

Investigations into other organisms capable of growing in environments with high arsenic and sulfide concentrations have revealed further examples of biogenic As–S nanowire formation. Strain TSA–1, a Citrobacter species isolated from the hindgut of the subterranean wood-feeding termite Reticulitermes flavipes (Kollar), grows in a freshwater mesophilic basal salts medium (Oremland et al., 1994) with cysteine-sulfide present as a reducing agent, and was originally isolated based on its Se(IV)-respiring ability. The organism was later found to have the ability to grow via H₂-driven respiration of As(V) as part of a study to evaluate arsenic speciation within the gastrointestinal tract (Herbel et al., 2002), with As (III) determined to be the product of this respiration. High-performance liquid chromatography analysis revealed the presence of a yellow thioarsenite precipitate (Color Plate 18A, inset) within the culture medium resulting from the presence of both arsenite (5 mM) and cysteine sulfide (0.5 mM). Electron micrographs (Color Plate 18A to C) showed that the As–S tube-like precipitates are several
micrometers long with a diameter of <100 nm and are composed of very small (~10 nm) crystallites. Scanning transmission electron microscope-EDS mapping images and spectrum (Color Plate 18C and D) confirmed the composition of the As-S nanotubes, with an As:S ratio suggesting that orpiment (As$_2$S$_3$) was the dominant phase. A mixed enrichment culture obtained from sediments of Searles Lake, California, using an extremely halophilic (salinity, 346 g/liter) and alkaliophilic (pH 9.5) culture medium (Searles...
Lake Ab1) was also investigated in terms of As(V) respiration and S(VI) reduction in extreme environments. This particular antibiotic-containing enrichment was, by design, a stratagem for the isolation of novel species from the domain *Archaea* with the ability to respire arsenate (Switzer Blum et al., 2009). An unexpected result of the antibiotic amendment was the proliferation in the culture of an S(VI)-reducing bacterium. The appearance of both an arsenate respirer and a sulfate reducer during the growth of this culture resulted in the formation of both arsenite (5 mM) and high levels of sulfide (5 to 10 mM). As(III) levels decreased at the end of the exponential phase of growth as thioarsenite species were formed. Figure 7C (inset) shows that the (yellow) precipitate formed under these conditions was substantially less dense than that observed with *Shewanella* sp. strain HN-41 (Fig. 6A, inset) and *Citrobacter* strain TSA-1 (Color Plate 18A, inset). This is to be expected when taking into consideration the pH of 9.5, at which thioarsenite species are expected to be soluble, with precipitation occurring under these circumstances as a result of the high arsenite and sulfide concentrations in the media. The less abundant As–S precipitates formed by Searles Lake Ab1 (Fig. 7A and B) were less filamentous and more structured than those formed by *Citrobacter* strain TSA-1 (Color Plate 18A), adopting a “nanoladder” morphology (Fig. 7A and 7B). The chemical composition of the As–S “nanoladders” was confirmed by EDS (Fig. 7C).

**CONCLUSIONS**

The ability of a large range of microbial species to produce nanoparticles relevant to one of the world’s most important technological frontiers makes their continued study of both intrinsic and commercial interest. In this chapter, we have presented some examples of these nanoparticles formed by only a few microbial species that are cultivated in only a handful of laboratories worldwide. Thus, the investigations so far have just scratched the surface of the potential of the natural world to yield biomineral producers. Indeed, examples of nanoscaled precipitates of group XV and XVI elements produced by representatives of the domain *Archaea* are entirely lacking. While future research should involve screening surveys of the prokaryotes for this biomineralizing...
phenomenon, more detailed investigations are justified. These should include more thorough physiological and biochemical investigations with known species, including variations in growth conditions and postpreparative techniques for the harvested nanoparticles. The ability to genetically manipulate the organisms (e.g., via knockout mutagenesis), especially if conducted with microbes having undergone full genomic sequencing and annotation, also holds promise to tune the fabrication of an enormous range of different nanoparticles by these novel biological pathways. The initial results highlighted here begin to reveal this potential for using a novel biological approach to produce functional nanoparticles, in what is an environmentally friendly methodology. A wide range of Se and Te nanostructures have already been identified that can be matched to several potential uses. The precipitation from bioselenide of capped, luminescent, semiconducting CdSe with chemical, optical, and electronic properties governed by composition, size, and shape takes this a step further, and direct comparisons with inorganically produced, similar materials can be made. The studies have also highlighted that novel biogenic fabrication routes can produce functional bielemental materials such as As-S nanostructures, for instance, for use in the construction of the next generation of nanoscale optoelectronic materials. However, the potential of bionanoparticle fabrication is largely untapped. Nonetheless, once novel Se, Te, and As bionanoparticles are identified as having significant technical applications, applied research into their practical commercial production will without doubt ensue rapidly.

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COLOR PLATE 17 (CHAPTER 16)  (A) Fluorescence spectra of size-fractionated glutathione-stabilized CdSe produced by *Veillonella atypica* (inset shows fractions 1 and 2 under UV light). (B) High-resolution TEM of glutathione-stabilized CdSe and accompanying EDX spectrum of fraction 1.
COLOR PLATE 18 (CHAPTER 16)  Electron micrographs of As-S precipitate formed by Citrobacter strain TSA-1 grown in the presence of arsenate in media with cysteine sulfide as a reducing agent and trace levels of sulfate (inset shows yellow precipitate). (A) SEM image. (B) High-resolution TEM image. (C and D) Scanning transmission electron microscope-EDX mapping images and spectrum (inset shows SEM image of strain TSA-1).
Chapter 3

Methodology

This chapter deals with the fundamental principles for a number of key analytical techniques used in the studies described in this thesis.
The fields of geomicrobiology and biogeochemistry are at the interface between the biological and chemical disciplines, and as such require a wide range of analytical techniques to fully characterise the effects these may have on experimental outcomes. This chapter aims to explain the workings of various techniques used throughout this research, and assess the usefulness with respect to the sample types analysed.

1. Electron optics

Electron microscopy provides imaging and analysis of materials below the resolution of standard optical microscopy techniques (typically >1μm), a limitation inferred due to the resolution achievable at the relatively long wavelengths of visible light, and is an invaluable tool in the analysis of sub-micron particles. The limitation of optical microscopy was first expressed mathematically by Ernst Abbé in the late 19th century in the equation below (equation 1.1), where $d$ is the smallest distance between two points that can be clearly resolved, 0.612 is Abbé’s constant, $\lambda$ is the wavelength, $n$ is the refractive index of the medium between the sample and the lens, and $\alpha$ is the half-angle of the light cone that reaches the lens from the specimen (9).

$$d = \frac{0.612\lambda}{n \sin \alpha}$$

*Equation 1.1. Abbé’s Equation*

As equation 1.1 shows, to decrease the distance between two clearly resolved points ($d$), the $\lambda$ should be minimised and therefore resolving images with high resolution at a higher magnification requires a smaller wavelength. Electrons provide a source of shorter wavelengths as described by quantum mechanics, and can be treated as a wave with a very small wavelength as opposed to a particle in classical physics, with a resultant resolving power of ~0.2nm (9). The mathematical relationship between the mass, velocity and wavelength of an electron is given in equation 1.2, where $\lambda$ is
equal to the wavelength, \( h \) is Planck’s constant, \( m \) is the mass of an electron and \( v \) is the velocity of the electron in volts (9).

\[
\lambda = \frac{h}{mv}
\]

*Equation 1.2. De Broglie Equation*

The interactions of electrons with a sample can be determined in a variety of ways; whether allowing the electron beam to flow through a sample with Transmission Electron Microscopy (TEM) or by scanning the electron beam across a sample’s surface with the Scanning Electron Microscope (SEM).

1.1. **Transmission electron microscopy**

Transmission electron microscopes are comprised of an electron gun and a series of electromagnetic lenses which serve to manipulate the flow of elections (figure 1.1). The electron source is typically an electron gun composed of a thin, pointed, tungsten filament which is heated to encourage the emission of electrons, although other materials such as lanthanum boride can be used (9). For nanoscale materials, it is necessary to use a high yield electron source such as a Field Emission Gun (FEG) to produce clear images due to the high resolution required. Around the filament is a Wehnelt cylinder, also subjected to the high voltage that drives the electron source and shields the electron source from the anode below and contains an aperture around 1-3mm in diameter centred on the tip of the filament (31). Below the electron source and Wehnelt cap (collectively the cathode) is the anode, which has another aperture to allow the passage of the electron beam. This anode is held in a grounded state, and it is the voltage potential between the anode and cathode that determines the energy of the electron beam (9). Condenser lenses are typically found between the sample and electron source, the exact number of these being specific to a particular instrument. The condenser lenses serve to control the
convergence point, coherence and the intensity of the electron beam prior to interaction with the sample (4).

*Figure 1.1. Schematic of a Transmission Electron Microscope (TEM). (After 4, 9, 31)*

Upon passing through the sample, the electron beam is refocused using an objective electromagnetic lens. This lens controls the resolution and intensity of the electron
beam that is received by the projector lens and subsequently by the detector apparatus (4). The detector apparatus is typically a fluorescent screen which interacts with the incoming transmitted electrons to release photons, or the image can be recorded on to film or digitally through a charge-coupled device (CCD) camera (9). At several points within the electron beam column are apertures that are designed to reduce noise that may affect the resulting image (figure 1.1): the column is kept under high vacuum (>10⁻⁴ torr) in order to allow the electron beam to propagate down the column without interacting with air molecules and so distorting the resulting image (9).

The interactions of the electron beam and the sample govern the nature of the image formed and these interactions are affected by numerous factors. The thickness of a sample plays an important role in determining the quality of the image obtained using this technique. The typical energy of the electron beam is between 60-100KeV, and at these energies electrons are easily absorbed or undergo large energy loss due to interactions with the sample. To counter this, very thin samples must be used, typically around 50-250nm in thickness (9). Alternatively, a very high energy electron beam may be used to image thicker samples; samples >5µm thick have been imaged using high-voltage electron microscopes (HVEMs) (9).

There are three possible outcomes for an electron passing through a sample:

i. it could pass through the sample completely unhindered,

ii. an electron could be deflected without significant loss of energy, also known as elastic scattering,

iii. an electron loses a significant amount of energy, referred to as inelastic scattering (27).

Elastic scattering occurs as a result of the interaction of the electron beam with the atomic nuclei of the sample material. The electron beam is diffracted by the nucleus, the level of diffraction proportional to the atomic mass of the element (figure 1.2) (3).
Figure 1.2. The relationship between sample mass, thickness and electron transmission. (After 3)

In figure 1.2, two sample parameters are examined, the atomic mass of a sample material and the sample thickness. Beams A and B pass through amorphous carbon, with beam A passing through 10nm of sample and beam B passing through 20nm. Carbon is a relatively light atom (atomic mass = 12) and only weakly scatters the electron beam, with only 9% of the beam electrons being scattered at an angle greater than 0.5°. At twice the sample thickness, the number of significantly diffracted electrons jumps to 17%. Beam C passes through 20 nm of amorphous lead (atomic mass = 207), and due to this large increase in mass, a much larger proportion of the electron beam (96%) is significantly diffracted. Beam D passes through 20nm of crystalline lead, but due to interference of electron waves travelling through the crystal lattice, most electrons will travel at angles of twice the Bragg angle (B°) relative to the incident electron beam. The proportion of electrons reaching the detector in this scenario depends greatly on sample thickness as well as the orientation of the crystal (3).

Apertures present within a transmission electron microscope are placed to remove diffracted electrons, with the objective aperture placed between the objective and projector lenses important in determining the contrast of a TEM image, as illustrated in figure 1.3 (3). Diffracted electrons are blocked from reaching the detector and so contribute to the formation of dark areas on an image, and so heavier elements present within a sample lead to darker areas on an image.
Inelastic scattering contributes to a TEM image through a process known as phase contrast. As an electron beam travels through a sample, a beam electron may interact with an electron of similar mass present within an atomic orbital of an element within the sample. Due to the previously mentioned wave-particle duality theory, in this scenario an electron may not be diffracted but could suffer a significant energy loss due to an alteration in the wavelength of the electron. Upon recombination of the scattered and unscattered electron waves, this may lead to destructive interference producing another source of variations in contrast in the resulting TEM image (3, 9).

In summary, TEM imaging is a valuable tool in the analysis of nanoparticles as a technique that is capable of clearly defining the morphological characteristics of precipitates. This capability of extreme magnification is however also a drawback in that the precipitation environment is not clearly indicated, and so a complimentary imaging technique is required to build up a full picture. Another complication with TEM is the high acceleration voltages used which may result in damage to the sample; amorphous Se\(^0\) precipitates have been seen to be particularly susceptible to damage and can readily crystallise if care is not taken.

1.2. Selected area electron diffraction

A secondary analytical technique that can be applied during the use of the TEM is selected-area electron diffraction (SAED). SAED can give valuable information on the structural characteristics of a mineral phase in a very small sample, and can relatively easily be used to obtain diffraction patterns from samples 1\(\mu\)m in diameter.
compared to the required samples of up to 20 times this for X-Ray Diffraction (XRD) (20, 31). Diffraction data from such small areas is possible due to the alteration in strength of intermediate lenses (present between the objective lens and projector lens, omitted for figure 1.1 for clarity) and the removal of the objective aperture so to allow the diffracted electron beam to pass. Another aperture, the selected area aperture, is introduced so to limit electron diffraction peaks to those occurring from within the area of interest (3).

![Electron Diffraction Diagram](image)

*Figure 1.4. Electron diffraction. (After 3)*

Similar to XRD, SAED occurs because of the diffraction of the electron beam by lattice planes within a crystalline structure (figure 1.4) (31). An incident beam striking a lattice plane at an angle $\theta$ will be reflected at an angle of $2\theta$ relative to the incident beam, and an electron diffraction pattern can occur when Bragg’s Law (equation 1.3) is satisfied, where $\lambda$ is equal to the wavelength, $d$ is the lattice plane spacing and $\theta$ is the angle between the lattice plane and the incident beam (and also the diffracted beam) so that multiple waves are running parallel and in phase with each other causing constructive interference (31).

$$n\lambda = 2d \sin \theta$$

*Equation 1.3. Bragg’s Law*

This technique is therefore useful for determining the structure of nanoscale precipitates and is an additional source of data that can be obtained during TEM analysis. SAED patterns obtained from crystalline nanoscale materials resemble rings.
rather than specific diffraction spots seen with larger crystalline samples, owing to the small scale of the particles. The diameter of the diffraction rings can be used to calculate the \(d\)-spacing of the diffraction plane, and compared directly with XRD information to determine crystal type.

1.3. **Scanning electron microscopy**

Scanning electron microscopes (SEMs) share many similarities with TEMs in the way in which electron beams are generated and manipulated prior to sample interaction. The major difference in the electron beam path prior to the sample is in the presence of scan coils within a deflection yoke (figure 1.5); variations in the current being passed through these electromagnetic coils alters the focal point of the electron beam in the plane of the sample (typically, x and y planes where the electron beam is propagating in the z-direction). In this way, the electron beam can scan across a surface point by point to build up a raster image of the sample (3).

The electron beam may interact with a sample surface in a variety of ways (figure 1.6) and the detector that is required is dependent on which of these emissions is to be analysed.

A relatively recent advancement in scanning electron microscopy has allowed for the analysis of hydrated, electrically non-conductive samples in a low vacuum environment. In a typical SEM, a high vacuum in the sample chamber is required in order to maintain the high vacuum necessary for the functioning of the electron column as well as to view high quality images by reducing interference signals. Samples viewed under a typical SEM also require dehydration and coating in a conductive material so to avoid ‘charging’; the persistence of a electrical charge on a non-conductive sample which can be re-emitted at a later time, appearing as bright streaks on an SEM image. This requirement limits the usefulness of this technique for biological and semiconductor applications due to the alterations of materials during dehydration and subsequent coating.
In the Environmental Scanning Electron Microscope (ESEM), a lower vacuum can be maintained in the sample chamber by the addition of several pressure-limiting apertures; small discs with holes drilled in to the centre to allow the electron beam to pass through. The small aperture size limits the diffusion of the low vacuum higher up in to the electron column, and so two different vacuums can be maintained within an ESEM using differential pumping (30). The Gaseous Secondary Electron Detector (GSED) forms the final aperture, the size of which therefore determines the strength of a vacuum with higher pressures maintainable with smaller aperture diameters (30). The requirement for a conductive coating is overcome by the introduction into the sample chamber of water vapour, which can be accurately controlled by a separate sample chamber vacuum pump. Figures 1.7,
1.8 and 1.9 illustrate the processes that allow for the formation of images in the ESEM.

![Diagram](image)

*Figure 1.6. Illustration of electron beam – sample interactions and depth of emissions. (After 31)*

As the electron beam excites a sample (figure 1.7), secondary electrons may be emitted from the surface of a sample (rarely exceeding 10nm depth (31)). These low energy electrons do not need line of site to the detector, as they can be manipulated via the addition of a current to attract or repel them, and so limiting the requirement for rotation of a sample (3). The current that is past across the GSED in the ESEM is used to control the contrast of the image, with the maximum contrast at the highest current, typically a 600 volt positive bias (30). The GSED is also physically mounted much closer to a sample than a standard secondary electron detector, and so is much more efficient at collecting secondary electrons (30).
Figure 1.7. Sample excitation by the incident electron beam in the ESEM.

(After 30)

The secondary electrons that are emitted from the surface of the sample collide with water molecules present within the ESEM sample chamber (figure 1.8). These water molecules subsequently emit further secondary electrons, producing a cascade of secondary electrons, which due to the positive bias on the GSED travels up to the detector (30).

Water molecules that have lost electrons due to the secondary electron cascade have obtained a positive charge (figure 1.9). A negative charge is acquired by the sample under bombardment with the electron beam due to the lack of conductive coating, and the two opposite charges therefore attract, further driven together by the repulsion experienced by the positively charged water molecules and the positive bias on the GSED. These two charges serve to neutralise the sample and limit the effects of charging, which may otherwise inhibit the images obtained by the ESEM.
Figure 1.8. Secondary electron cascade by electron emission from water vapour molecules within the ESEM. (After 30)

Figure 1.9. The negative charging of the sample by the incident beam is countered by the attraction of positively charged water vapour molecules. (After 30)

There are however disadvantages of using the ESEM over a traditional SEM. The magnification and resolution of an ESEM is lower than that of an SEM owing to the lower required vacuum and higher humidity, and the lack of conductive coating over the sample means that although steps have been taken to prevent it, charging of a sample still occurs. The imaging of nanomaterials on the ESEM is also limited owing to the extremely small size of the particles, however ESEM can be considered to be
complimentary to TEM imaging in that it provides an insight into the location and vague morphology of the precipitates.

1.4. **EDXS**

Energy Dispersive X-Ray Spectroscopy (EDXS) is the analysis of characteristics X-rays emitted as a result of the interaction between the electron beam and the sample (figure 1.6). Characteristic X-rays are produced as a result of the emission of electrons from the sample and the subsequent relaxation of higher orbital electrons with the associated photon emission. Due to the quantized nature of electron shells it is possible to determine the original element emitting a photon, although this technique becomes less sensitive with the lightest elements due to the tendency of the light elements to emit Auger electrons rather than photons with relaxation. A typical EDXS detector is composed of a thin (~7.5µm) beryllium window through which the X-rays pass into a high vacuum chamber containing a lithium-drifted silicon crystal, and is typically cooled down to liquid nitrogen temperatures so to reduce electronic noise (41). The absorption of an X-ray by the Si(Li) crystal leads to the emission of an electron whose energy is proportional to the incident x-ray energy minus the known binding energy of the electron orbital (41). The intensity of the emission can then be plotted against the photon energy to give an elemental spectrum for the sample (figure 1.10).

![Figure 1.10. An ESEM-EDX spectrum of Te\(^0\) precipitates.](image)

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EDXS equipment may be attached to SEMs, ESEMs and TEMs. The instrument to which the EDXS is attached will dictate the spot size for analysis, with TEMs coupled with a high electron yield FEG capable of producing EDX spectra of nanoparticles owing to the high magnification and resolution of this technique. EDXS attached to ESEMs provide a valuable tool to analyse in situ biological materials that have undergone a limited amount of dehydration, and are so suited to larger precipitates and biofilms.

EDXS is a not oxidation state specific, and the observed spectra must undergo a number of corrections including background subtraction and the likelihood of backscattering of the incident beam, the likelihood of re-absorption of a photon and the likelihood of emission at atomic mass (ZAF corrections).

2. X-ray techniques

2.1. X-ray absorption spectroscopy

The analysis of samples by synchrotron based X-ray Absorption Spectroscopy (XAS) can give detailed information on the atomic environment of the sample owing to the interaction of the incident photons with a specifically targeted element. Numerous synchrotron radiation facilities are located around the world; this research project has specifically used three facilities: (i) the high brilliance X-ray spectroscopy beamline ID26 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France, (ii) the microfocus spectroscopy beamline I18 of the Diamond Light Source, Didcot, UK and (iii) the now decommissioned Synchrotron Radiation Source (SRS) Daresbury laboratories, Warrington, UK.

X-rays are generated within a synchrotron by variations in the path of electrons around a storage ring (figure 2.1), which are then channelled to an end station where a sample is analysed. A monochromator crystal allows for the selection of a particular wavelength of X-rays, and alterations in the orientation of this crystal allows for a consistent variation in the energy of the X-rays reaching the sample, therefore allowing the energy to sweep across the absorption edge of an element and into the EXAFS region.
Figure 2.1. X-rays derive from an alteration in the path of a beam of electrons within a synchrotron storage ring (A) by means of strong electromagnetic undulators or ‘wigglers’ (B), and these x-rays are channelled (C) and focussed by monochromator crystals (D) in the beamlines which radiate out from the storage ring, and into a sample held within an end station (E)

XAS techniques rely on the absorption of an X-ray by a target atom at or slightly past its excitation potential, resulting in the emission of an electron and creating an electron hole. This hole is then filled by an electron from a higher shell, with excess energy emitted either as a photon or an Auger electron, the energy of which corresponds to the binding energy of a particular shell of a particular element (figures 2.2, 2.3 and 2.4).
Figure 2.2. A photon of sufficient energy hits an electron in the absorbing atom, causing the excited electron to either jump to a higher, vacant shell or to exit the atom entirely, leaving behind a vacancy in a low orbital. The excess energy of the excited electron is equal to the energy of the incident beam minus the binding energy of the electron in a specific shell of a specific element, and so is element specific. The energy required to excite an electron increases with decreasing distance between the nucleus and the electron shell (6).
Figure 2.3. The vacancy left behind by the excited photoelectron will be filled by an electron from a higher orbital. Electrons in higher orbitals have a higher energy state, and when dropping down to a lower orbital will release this energy in the form of electromagnetic radiation. This fluorescence can be registered and used as a measurement of the absorbance (38).

Figure 2.4. An alternative to the emission of electromagnetic radiation is the emission of an auger electron, which has an increasing possibility of occurring with decreasing atomic weight. The excess energy emitted by the transition of an electron from a higher orbital to fill a vacancy in a lower orbital may be transmitted to a second electron, which is in turn excited to a higher orbital or past the Fermi level (33).

XAS spectra can be recorded in a variety of ways. The classical XAS method compares the intensities of the incident beam with that of the transmitted beam, the difference between the two being the absorption (figure 2.5). These two factors are related in equation 2.1, where \( I_t \) is the transmitted portion of the beam, \( I_o \) is the incident beam, and shows that the absorption of a sample is driven by the thickness of the sample (\( \chi \)) and the absorption of X-rays per unit distance (\( \mu \), the linear absorption coefficient) (11).
Alternatively, XAS spectra may be recorded by measuring the intensity of fluorescence or by measuring the current generated by emitted Auger electrons (figure 2.5).

\[ I_T = I_0 e^{-\mu x} \]

*Equation 2.1.* The relationship of the intensity of the transmitted beam to the incident intensity. (After 11)

*Figure 2.5.* The apparatus setup as seen at the CCLRC SRS Daresbury, Warrington, UK. The beam is represented as the solid black line, with arrows indicating the direction of propagation. A and C are the pre- and post-slits, respectively. B is the monochromator mechanism. D and F are ionisation chambers for the quantification of \( I_0 \) and \( I_T \). E is the sample. G is a fluorescence detector for the measurement of XAS in dilute systems, positioned at a 90° angle to the incident ray and a 45° angle to the sample (After 10).

XAS spectra contain two regions of interest that describe the oxidation state of the sample (XANES) and the atomic environment around the absorbing atom (EXAFS) (figure 2.6). The X-ray Absorption Near Edge Structure (XANES) region begins at the
absorption edge and extends ~50eV up to the beginning of the Extended X-ray Absorption Fine Structure (EXAFS) region, extending for up to a further 1000eV (6). Work by Bianconi (1) indicates that the dividing energy between the XANES and EXAFS regions is where the wavelength of the excited electron is approximately equal to the distance between the absorbing atom and its closest neighbouring atoms.

![Figure 2.6. A simplified XAS plot identifying the XANES and EXAFS regions (After 6)](image)

XANES spectra can provide detailed analysis of the oxidation state of the target element, as well as the spatial arrangement of atoms near to the absorbing atom, showing their radial distances as well as orientations and bond angles (6). This information arises due to the effect of multiple scattering, where the limited excess energy of emitted photoelectrons are strongly influenced by many of the neighbouring atoms, as well as by many other factors such as the nature of the bonding to neighbouring atoms and the presence of diatomic molecules (6). These effects greatly increase the difficulty in analysis of data obtained by XANES, and so
this technique is typically employed for elemental ‘fingerprinting’ and oxidation state discrimination. This region is of notable importance with Se samples as there is a large difference in absorption edge energy (~8eV) between the reduced Se$^{II}$ and the most oxidised Se$^{VI}$ phases, meaning this technique is well suited to the identification of unknown Se forms within an environmental substrate. XANES analysis is also well suited to the identification of Hg mineral phases due to the presence of ‘shoulders’ in the $L_{III}$ absorption edge; 1$^{st}$ and 2$^{nd}$ order derivatives of the XANES spectra show differences in the inflection points according to mineral phase present, with even differences between allotropes of HgS (cinnabar and metacinnabar) discernable (14, 15).

EXAFS information is comparatively easy to extract from XAS spectra, and analysis and interpretation of EXAFS data has been ongoing for several decades (6, 10). EXAFS oscillations arise as a result of interference between a propagating electron wave emitted due to the absorption of a photon 10s of eV above the absorption edge (thus decreasing the effects of oxidation state, as in XANES), and the backscattered wave rebounding from neighbouring atoms (figure 2.7) (38).

\[\text{Intensity} \quad \text{Energy}\]

\[\text{Constructive} \quad \text{Destructive}\]

*Figure 2.7. The origin of EXAFS oscillations by constructive and destructive interference. (After 38)*
As the wavelength of the emitted photoelectron is inversely proportional to the energy of the incident X-ray, then the wavelength should steadily decrease as the energy of the emitted photoelectron increases, and so oscillations should develop in the EXAFS spectrum due to the backscattered electron wave repeatedly moving in to and out of phase with the propagating wave (10).

EXAFS oscillations are plotted as a function of the photoelectron wave vector (equation 2.2), and the resulting plot can be used to determine the distance to the neighbouring atom by means of a Fourier transform (figure 2.8), and can then be compared against known standards (10).

\[ k = \frac{2\pi}{h} \left[ 2m(E - E_O) \right]^{1/2} \]

*Equation 2.2.* The EXAFS equation. (After 10) Where \( k \) is the photoelectron wave function, \( h \) is Planck’s constant, \( m \) is the mass of the electron, \( E \) is the energy of the X-ray photon and \( E_O \) is the energy of the absorption threshold.

*Figure 2.8.* Example EXAFS plot (left) and resulting Fourier transform (right) (After 10).

Synchrotron radiation sources are ideal for the analysis of dilute samples of solid and liquid materials owing to their high brilliance, and with this research project have been applied to investigate Se concentrations in a number of forms including dilute aqueous solutions of biogenic selenide and solid environmental samples. As a result
of the low concentrations, synchrotron XAS experiments are typically undertaken using external fluorescence detectors mounted perpendicular to the X-ray beam so to increase the signal:noise ratio. From a practical perspective, XAS techniques pose a problem unique to anaerobic samples in that the apparatus is rarely designed for maintaining anoxic atmospheres. Accordingly, specialised anaerobic cells have been used, and extensive care was taken to ensure samples (with special reference to biogenic Se\textsuperscript{II}) were not exposed to atmospheric O\textsubscript{2}.

2.2. X-ray fluorescence

The principle behind X-Ray Fluorescence (XRF) is similar to the characteristic X-ray emission as previously described, as these X-rays arise due to the relaxation of an element from an ionised state due to the loss of an inner shell electron (35). The loss of an inner shell electron may be triggered by either the absorption of x-rays such as in X-ray Absorption Spectroscopy (XAS) or via collision with incident electrons, as with electron microscopy. Typical X-ray emissions are shown for the inner orbitals (figure 2.9) and describe how photon emissions are categorised by the atomic orbital into which the electron enters (eg K\alpha1 emission).

Figure 2.9. The electron orbital transmission.
XRF is a relatively quick and simple technique that can provide qualitative and quantitative multi-elemental analysis without chemically altering the sample (35). This technique however is not suitable for use in the analysis of the lighter elements due to the increasing likelihood of auger electron emission (35).

XRF was used in this project to analyse the chemical composition of soil cores collected from field sites in Ireland. Aliquots of horizons taken from the soil cores were dried and powdered prior to being pressed in to pellets for loading directly in to the XRF.

2.3. X-ray diffraction

X-Ray Diffraction (XRD) is one of the key analytical techniques for providing information on the crystallinity of a material, and is a useful fingerprinting tool. Minimal sample preparation is required, usually consisting of fixing a powdered sample to a holder prior to bombarding with Cu Kα X-rays. Incident X-rays may be diffracted by the surface lattice planes of a crystalline material at an angle twice the angle of incidence (θ, figure 2.10).

![Figure 2.10](image)

*Figure 2.10. A simplified illustration of incident X-rays diffracting from a set of lattice planes within a crystalline structure. (After 35)*
If one of the three waves shown in figure 2.10 is diffracted off the top most lattice plane and another is diffracted from a lattice point further within the crystal, then the latter wave will have travelled a greater distance and so the two electromagnetic waves may no longer be in phase. This is illustrated in figure 2.10 where beam 1 has been diffracted from the surface lattice plane, and beams 2 and 3 have been diffracted from the second and third lattice planes, respectively. Beam 2 has travelled the extra distance $A \to C$ relative to beam 1 and beam 3 has travelled the extra distance $B \to D$. This extra distance can be determined empirically by the analysis of the interference on the diffracted waves, and where the extra distances travelled are equal to an integral number of wavelengths, constructive interference occurs and a peak in diffracted X-ray intensity is recorded. These extra distances travelled are proportional to the $d$ spacing of the lattice planes and the angle of incidence, which is defined by the orientation between the X-ray source, the sample stage and the detector. For X-ray powder diffraction, simply rotating the X-ray source and the detector about the sample (keeping the angle between the X-ray source and detector fixed to $2\theta$ relative to the angle of incidence) will satisfy most possible angles and examine all lattice planes and so provide a near complete XRD spectrum for a sample. The $d$ spacing, the wavelength and the angle of incidence can all be calculated using the Bragg equation (equation 1.3) (35).
Figure 2.11. An example XRD plot for the iron oxyhydroxide lepidocrocite. (5)

The recorded peaks in intensity at particular 2θ values (also represented as θ values or d spacing) can then be compared to records of known materials to identify the sample being examined, and relative peak intensities can be used to determine the crystal form of a sample, particularly whether any single or set of crystal faces are dominant; more dominant lattice planes will have higher intensities on an XRD spectrum (figure 2.11).

3. Ion chromatography

Ion chromatography is used in the analysis of ionic constituents of aqueous solutions, relying on the adsorption and desorption of charged molecules. An aqueous sample can be analysed by passing through a column containing a stationary phase, a solid material to which are fixed polarised functional groups, either positively or negatively charged dependent on the analysis of anions or cations, respectively (42).
A mobile phase containing ions which can exchange with the analyte is then passed through the column, resulting in desorption of the analyte according to equilibrium between the stationary phase, the mobile phase and the analyte (figure 3.1). By varying the flow rate and concentration of the mobile phase it is possible to separate desorption of polar groups within the sample (i.e. vary the retention time for different phases), and comparison against known standards allows for identification (42).

Detection of the analyte can be achieved by a number of methods; for this project both the conductivity and the UV-vis absorption of the eluent were measured owing to the particular chemical characteristics of Se and Te, respectively. Quantification of the concentration of the analyte can be achieved by comparison with known standards.
4. Inductively coupled plasma

Inductively Coupled Plasma (ICP) techniques rely on the ionisation of acidified samples prior to analysis, and require the addition of a second detector module for element identification, commonly an atomic emission spectrometer (ICP-AES) or a mass spectrometer (ICP-MS). These techniques are not sensitive to oxidation state due to the ionisation of the analyte, however are well suited to the accurate quantification of elemental abundances in a sample, commonly down to ppb levels.

With an ICP, the primary step is the generation of the plasma. This is achieved with argon gas at a flow rate of ~17 L min⁻¹, which is ionised by a tesla coil (2). The ionised argon interacts with a fluctuating magnetic field produced by water-cooled radiofrequency coils (figure 4.1), where the temperature reaches up to 10,000°K by ohmic heating (35). The acidic solution of the sample is introduced to the ICP as an aerosol dispensed from a nebuliser, and is carried up through the central tube of the ICP via an argon carrier gas, where it becomes ionised through bombardment with the charged argon particles and free electrons.

With ICP-AES, the repeated ionisation and recombination of charged particles leads to the emission of photons with wavelengths characteristic of specific electron orbital transitions, which are separated with monochromator crystals prior to detection at a specific predetermined wavelength for a desired element. The intensity of the emission is proportional to the concentration of the element within the sample, which can be calculated with the use of appropriate standard solutions.
For more accurate determination, ICP-MS can be used to detect analyte concentrations as low as 10s of ppt (for example, Hg). In contrast to ICP-AES, the charged analyte particles are accelerated away from the plasma flame using electromagnetic fields towards a mass spectrometer, again set to predetermined requirements to detect particle collisions of a specific mass.

Both ICP-AES and ICP-MS are invaluable tools for the determination of low concentrations of aqueous Se and Te phases. With Se especially, ICP techniques aid in following the transfer from one oxidation state to another, as when used in conjunction with other aqueous phase analytical techniques (notable ion chromatography) a full catalogue of inorganic Se phases can be determined.
5. **Spectrophotometry**

Spectrophotometry is a technique for determining the concentration of a coloured compound in solution or biomass in suspension by measuring the absorption or scattering of light at a specific wavelength. The technique is commonly used for the quantification of iron in solution with the chromophore Ferrozine (39), which can be calculated with the aid of standards of known concentration.

*Figure 5.1. A simplified diagram of a spectrophotometer. (After 11)*

In a UV/Vis spectrophotometer (figure 5.1), two lamps are used to generate wavelengths that extend from the ultraviolet portion of the electromagnetic spectrum through to visible light. Tungsten filament lamps in conjunction within deuterium lamps for the UV emissions are simultaneously emitting light, which is separated into the wavelengths of interest by a monochromator. The light then reaches a half mirror, which reflects some to a photodiode to determine the original intensity of the light ($I_0$). The remaining portion travels through the sample which is commonly housed in a low-absorbance disposable plastic cuvette, and then the intensity is recorded by a photodiode on the far side ($I$) (11). This arrangement allows for the removal of errors generated by variations in light intensity over time. For quantitative data, a reference solution is required, which is typically the medium into which the analyte is placed; unreacted ferrozine for ferrous iron determination, sterile media for cells in media or buffered solution for washed cell suspensions. For
correlation of the recorded absorbance to a solute concentration, a standard curve must be recorded and plotted, and for justifiable results the data set must lie within the linear portion of a standard curve.

The theory behind spectrophotometry is based on the Beer-Lambert law (equation 5.1), which expresses the relationship between the absorbance of a substance (A) with the thickness of a sample (l), the molar absorption coefficient at a specific wavelength of light (ελ) and the concentration of the chromophore (c) (11).

\[ A = \varepsilon_{\lambda} cl \]

*Equation 5.1. The Beer-Lambert Law*

For cell culture quantification, the absorbance of a sample is not the principle cause of the difference between \( I_o \) and \( I \); this difference is due to the scattering of light by cells and is why the attenuation (D) or optical density (\( OD_\lambda \)) of a cell suspension is referred to rather than an absorption (11). In this case, the attenuation of a suspension is recorded (equation 5.2) (11).

\[ D = -\log_{10} \left( \frac{I}{I_o} \right) \]

*Equation 5.2. The relationship between the incident intensity, transmitted intensity and the attenuation.*
6. Microbiological techniques

6.1. Epifluorescence microscopy

Epifluorescence microscopy is a technique where a sample is stained using a specific fluorescent stain (fluorophore), commonly acridine orange, which is excited at wavelengths of 502nm with an emission maximum of 526nm (green) (29). An aliquot of a cell culture fixed in a glutaraldehyde and ammonium oxalate solution is filtered onto a 0.22μm micropore filter. An acridine orange solution can then be applied to the filter, where it permeates across cell membranes, binding to DNA and RNA. The filters containing the stained cells are then mounted on to glass slides with a cover slip, ready for examination under the epifluorescence microscope.

Light emitted from a xenon or mercury arc lamp, which are used due to being able to emit wavelengths extending from UV to red visible light, is passed into the epifluorescent microscope where it is reflected on to the sample by a dichromatic mirror and through the objective lens (figure 6.1). This method of illuminating the sample from above by using the objective as both the objective and condenser lenses is why epifluorescence microscopy is differentiated from fluorescence microscopy, as this technique removes background noise due to excess light being transmitted through the sample away from the eyepiece or CCD detector (29). Sample fluorescence due to fluorophore reactions result in the light emitted travelling back up the objective lens, where this longer wavelength passes through the wavelength-selective dichromatic mirror. The light then passes through an emission filter, which only allows the passage of light in a particular, narrow range of wavelengths through; the emission filter is specifically chosen for the fluorophore which is used (29).
The image formed, composed of green fluorescent cells on a black background (figure 6.2), can now be viewed either through an optical eyepiece or via a CCD detector mounted to a digital camera or computer.

The cells as viewed under the epifluorescence microscope can now be counted with the aid of a 10x10 grid engraved into an eyepiece. This grid has been superimposed over an image of a haemocytometer grid (figure 6.3) to indicate the area this grid represents. The 10x10 grid covers the same area as four of the smaller grid squares on the haemocytometer, 0.01mm². This is then comparable to the total area of staining on the black filter, typically ~15mm in diameter, and can be used to determine the number of cells present in the original 0.1ml of cell suspension.
Figure 6.2. The view down the microscope at 1000x magnification using Acridine Orange. The cells are clearly visible as the light green specks.

Figure 6.3. The eyepiece 10x10 grid overlying the grid squares on a haemocytometer to indicate the scale of the grid. Photographs of the actual 10x10 grid are not achievable due to the grid being etched on an eyepiece out of the line of sight of the digital camera.

Epifluorescence microscopy is used when it is necessary to directly determine a direct count of the number of cells in a sample, rather than by proxy using spectrophotometric methods of optical density and total protein content, or by
comparatively inefficient plate counting or culture-dependent dilution series techniques in microbial ecology studies. This technique is also required when solutes are present within microbial cultures which are capable of interfering with protein assay components, such as the oxyanions of Se and Te, and when particle precipitation negates the use of optical density determination, such as with Se⁰ and Te⁰ precipitates.

6.2. Microbial ecology by TRFLP

For this research project, changes in the microbial community over time and under varying geochemical conditions was followed by statistical analysis of Terminal Restriction Fragment Length Polymorphism (TRFLP) digests of 16S rRNA gene extracts amplified by the Polymerase Chain Reaction (PCR). There are several stages involved in the assessment of changes in the microbial community which are outlined in brief below.

i. DNA extraction

The first step in microbial community analysis is the extraction of genetic information from an environmental sample, requiring both the separation of the DNA/RNA from within a cell and purification, removing any contaminants which may affect further processing. Commercial kits exist which contain patented mixtures of chemicals, such as the PowerSoil DNA Isolation Kit (MO BIO Laboratories INC, Solana Beach, CA, USA), although all rely on similar principles.

Environmental soil samples are mixed with small glass beads, sodium dodecyl sulphate (SDS) and a buffer solution and shaken vigorously. The glass beads and the anionic surfactant SDS help to break open and dissolve cell membranes, whilst the buffer solution prevented decay of the genetic material (25). The solid materials are separated from the solution via centrifugation, with the supernatant containing the DNA transferred to a new sterile eppendorf.

The supernatant bearing the DNA is mixed with a number of reagents designed to precipitate soluble contaminants, which are removed sequentially by centrifugation. The remaining solution is transferred into a spin filter; a centrifuge vessel fitted with
a silica membrane to which DNA will bind (25). A series of washes are then undertaken using this spin filter, including washes to remove humic substances with the resulting solution discarded each time. A final wash with a supplied elution buffer releases the bound DNA from the silica membrane, and the resulting solution is then flash frozen and stored prior to PCR amplification.

**ii. PCR**

The Polymerase Chain Reaction (PCR) is a molecular technique designed to amplify a selected gene or region of genetic code of interest from a small quantity of DNA extract. Many detailed descriptions and reviews of PCR have been published, including those by Innis and Gelfand (16), McPherson *et al* (23) and Mullis *et al* (26). PCR is a repeated cycle of reactions whereby an initial strand of genetic information is unravelling and copied, increasing the concentration of DNA/RNA to a level where it can be used in further experiments (see figure 6.4). The initial step involves denaturing the DNA double helix, breaking the hydrogen bonds that exist between the base pairs on either strand of the DNA molecule. Once the DNA strands have been separated, short oligonucleotide ‘primers’ can be attached; for this research project, the 8f (8) and 1492r (17) primers were used, which bind to complimentary strands of the highly conserved bacterial 16S rRNA gene region. The primer-DNA strands are then allowed to anneal with fresh nucleotide base pairs, using the enzyme *Taq* polymerase (7) to synthesise a complimentary DNA strand running between the 8f and 1492r primers. Repeated cycling of these denaturing and annealing steps allow for the increase in concentration of the genetic sequence of interest (the ‘short’ product, figure 6.4), as well as less concentrated, longer sequences of genetic information formed from the initial full-length DNA extract.

**iii. TRFLP digest, TRF detection and alignment**

The PCR products can now be used in terminal restriction enzyme digests, a technique known as Terminal Restriction Fragment Length Polymorphism (TRFLP). As with PCR, there is an extensive amount of literature dealing with TRFLP (13, 19, 21, 22, 24, 28, 32, 36, 40).
Figure 6.4. PCR amplification of 16S rRNA using 8f and 1492r primers. (After 43)

The fundamental principle behind TRFLP is the use of restriction enzymes to cut the genetic sequence at a particular point. There are a large number of available restriction enzymes, including *Hha I, Msp I, Rsa I, Hae III*, and a number of restriction
enzymes are usually used in parallel experiments to ensure the accuracy of the results by cross-referencing datasets. For use with TRFLP, it is necessary that primers used in the PCR step were fluorescently labelled; in this research, the 8f primer (8) was labelled with 6-FAM (6-carboxyfluorescein, Eurofins MWG Operon, London, UK) and the 1492r primer (17) was labelled with HEX (hexachlorofluorescein, Eurofins MWG Operon, London, UK). The fluorescently labelled PCR products were incubated with the restriction enzymes according to the manufacturer’s instructions, where the enzymes bound to their specific target areas and cleaved the DNA strand (figure 6.5). Following digest, the cleaved Terminal Restriction Fragments (TRFs) were mixed with an internal size standard, which served as a reference for the samples. In this research, the size standard LIZ 1200 was used (Perkin Elmer-Applied Biosystems, Warrington, UK). The TRF digest was analysed using an ABI Prism 3100 Genetic Analyser, a capillary electrophoresis sequencer which detects fluorescent emission of samples. The only sources of fluorescent emission within a sample are the fluorescently labelled forward and reverse primers and the size standard, and so only the DNA fragments at either end of the sequence of interest are detected. Furthermore, as the forward and reverse primers fluoresce at different wavelengths, it is possible to discern fluorescent emission from each primer set. The raw data can be analysed using ABI PeakScanner software, producing a plot showing the relative fluorescent intensity vs. the TRF size (figure 6.5). It is then possible to remove fluorescent emission below a specified threshold from the data set, thus increasing the signal:noise ratio, and to remove the contribution to total fluorescent intensity from emissions below 50 bp in length, which are contributed by unreacted primers. The final stage prior to statistical analysis of the TRF profiles for the samples is to align replicate sample profiles, removing any peaks which do not occur in all samples and so removing a source of error. This is accomplished using the software T-Align (37), which outputs a series of text files containing (i) a consensus file, listing the intensity of all TRFs detected for a sample, (ii) a comparison results file listing the relative intensity of all TRFs detected for all samples and (iii) a binary comparison results file, stating whether or not a TRF is present in a particular sample.
Figure 6.5. Assessment of the microbial community via TRFLP. The steps of the restriction enzyme digest are shown above, with a TRF profile of a model sample shown below; the blue peaks represent 6-FAM labelled 8f forward fragments, the green peaks HEX labelled 1492r reverse fragments and the orange peaks the size ladder used for referencing. The red shaded area is the region below 50bp which must be discarded to remove erroneous results, as suggested by running blank samples.

iv. Multivariate ordination analysis
The TRFLP results files generated by the previous step can now be used to statistically analyse the microbial community for each sample.

The analysis, representation and interpretation of multivariate datasets can be simplified greatly with the use of ordination methods, whereby data requiring many axes can be condensed to a single 2-dimensional plot displaying the similarity between samples, with samples appearing closer together displaying a higher similarity (18). A further consideration is the measure of similarity used; there are a number of similarity and dissimilarity measures including Jaccard, Bray-Curtis, Sørensen and Euclidean. The Bray-Curtis similarity measure was chosen for this research as it accounts not only for species presence-absence but also for individual species abundance (18). With the aim of comparing microbial community similarity across samples, there are no predictor ‘explanatory’ variables present (i.e. variables that can be used to predict species found), which therefore reduces the total number of ordination methods available. Non-metric multidimensional scaling (NMDS), a form of indirect gradient analysis, and cluster analysis were used to interpret the TRFLP profiles produced (figure 6.6). A number of software packages exist that can be used for statistical analysis of microbial communities, and the ecological statistics package PAST (12) was chosen for this research.

TRFLP is an advanced molecular microbial ecology technique that can be used to assess changes in the microbial community across a sample set, and can even attribute changes in microbial community with changes in geochemistry, should an experiment be created to view this.

There is a significant caveat that needs to be addressed to ensure the robustness of the obtained dataset; the fluorescent emission peaks which form the basis of the statistical analysis assume that each TRF peak is representative of an individual bacterial species or genus. This may not be the case, as it is possible for a number of disparate bacterial species to share a TRF peak, owing to the finite length of the 16S rRNA gene region. To counter this, it is necessary to use either fluorescently labelled forward and reverse primers, multiple restriction enzymes or preferably both, as this greatly reduces the likelihood of two distinct bacterial species sharing both forward and reverse TRF lengths for multiple restriction enzymes. It is then possible to
correlate datasets across restriction digests and discard any that show little dissimilarity between samples.

**Figure 6.6.** Sample NMDS plot (left) and cluster analysis dendrogram (right) taken from the Co. Meath field site (Chapter 4) using the Bray-Curtis similarity measure and the Msp I restriction enzyme and the 1492r-HEX primer. The key for the NMDS is (cross) t0, (open circle) t4, (solid circle) t4 + Se, (open square) t4 + NO₃, (solid square) t4 + NO₃ + Se.

Finally, it is possible to cross reference multiple restriction enzyme digests for a sample against a database of known, fully sequenced bacteria to produce a list of bacterial species present (34). For this, an *in silico* PCR and restriction digest using selected primers and restriction enzymes is matched against the empirical data obtained above, and a likelihood of presence for each bacterial species is given dependent on the presence or absence in all restriction digests and for all primers used. This final step however still requires considerable development, as the primary databases used for the *in silico* digests contain a significant bias towards bacterial species characterised within medicinal and biochemical research, disproportionate to environmental microbiology. Thus, data outputs from these modelling programs may erroneously match bacteria with TRF peaks, creating misleading results. For this reason, identification of bacteria via TRFLP was not explored further within this research project.
References


Chapter 4

Research paper 1

Microbial cycling of selenium in seleniferous soils

Microbial Selenium Transformations in Seleniferous Soils


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Abstract

Selenium is an essential trace element with a narrow range between dietary deficiency and toxicity. The toxicity of Se depends on its bioavailability, which is directly related to its oxidation state, of which four occur in the environment (SeVI, SeIV, Se0 and SeII). The cycling of Se between these oxidation states is driven by microbial communities. In order to investigate the effect of microbial activity on Se cycling in the environment, a field site in County Meath, Ireland, was identified with anomalously high levels of Se as a result of weathering of black shales within the Lucan formation, leading to cases of Se toxicity in farm animals. Soil cores were extracted from the site for Se speciation and microbial community analysis prior to microcosm experiments to assess Se stability and microbial Se transformations. Se was present as a recalcitrant, reduced organic phase that was strongly coordinated with carbon, concordant with suggested hypotheses of Se phytoconcentration within a clay-lined, postglacial marshland.
Se was not mobilised in microcosm experiments, and supplementation with SeVI resulted in rapid reduction and removal from solution as Se0. Additional electron donors did not affect Se stability or removal from solution, although nitrate did hinder SeVI reduction. The microbial community notably changed following the addition of SeVI, suggesting bacterial species present were not highly resistant to soluble Se. This work extends the current knowledge of Se cycling in the environment, and provides information on the bioavailability of Se in the soil, which determines Se content of foodstuffs. Furthermore, seleniferous soils represent an area to focus the search for microbial species capable of producing industrially and technologically useful materials via ‘green’ synthetic pathways.

**Key words:** selenium, environmental cycling, bioremediation, seleniferous

1. **Introduction**

Selenium (Se) is an essential trace nutrient, present in a range of seleniferous proteins and enzymes but the small range between dietary deficiency (<40µg day⁻¹) and toxicity (>400µg day⁻¹) requires careful control, especially when monitoring human diets and supplementing feedstuffs for livestock (5, 24, 53, 65). The bioavailability and, thus, toxicity of Se is largely dependent on local geochemical conditions. In aqueous solution, such as groundwater and pore waters, Se is commonly found as the SeIV and SeVI oxyanions, with the latter being predominant and mobile under more oxic, alkaline conditions (24, 35, 45). Under reducing conditions, insoluble Se0 and SeII mineral phases are expected (24, 29, 35). Volatile organic species, such as dimethylselenide, are also found in soils and contaminated ground and formed as a result of biotic methylation (13, 40). Biological transformations are a major driving force in the change of Se from one species to another, and a variety of biochemical mechanisms are responsible for the large range of seleniferous compounds found in the environment (35, 38, 60, 61).
Globally, soil Se concentrations are low, typically between 0.1 and 2.0 μg g⁻¹ (4, 27, 62). Cultivation of Se-poor soils has led to reports of Se-deficiency diseases such as Keshan disease (7, 25, 54). Amendment of Se to agricultural soils in Se-poor areas such as Finland has lead to an increase in Se dietary uptake, with Se being found within crops as a wide range of organoselenium compounds (6, 17, 18, 38). The Se content of a soil is thought to be largely attributable to the Se content of the underlying lithology (24). Organic-rich rocks, such as coal measures and black shales may contain elevated Se concentrations, and weathering products of these rocks coupled with enrichment mechanisms such as evapo- or phyto-concentration can produce soils that contain Se in to the mg g⁻¹ range (23, 24, 35, 50). Se toxicity in both animal and human populations has been reported in areas of highly seleniferous soils (9-11, 26, 52, 55, 68) and these areas can be separated by as little as 20km (26).

Cases of Se toxicity in animals in Ireland date back to the late 19th century (46), and Rogers et al (55) list 7 counties affected by high soil Se concentrations. Fleming et al (22) identify a field site near Trim, County Meath, with a soil Se concentration of 1200ppm. The origin of the Se is reported to be the interbedded muddy limestone/shales of the Calp Limestones, part of the Lucan Formation (42, 46). Weathering of the seleniferous rocks by alkaline drainage waters favour the formation of mobile Se⁶⁺ oxyanions, which are transported into low lying, clay lined basins, formed as a result of glaciation during the last ice age (22, 42, 46). Poor drainage results in the development of low lying marshland, allowing Se to accumulate.

In addition to its role as a nutrient, selenium is utilised in a wide range of chemical and technological applications owing to its unique chemical and photo-optical properties, including applications in photovoltaics, semiconductors, rectifiers, dye reduction, steel and ceramic manufacturing and sequestration of hazardous wastes (3, 20, 31, 32, 45, 47, 69). Understanding the natural microbiological pathways developed to processes high selenium in the environment provides the opportunity to control its bioavailability and to synthesise selenium-based materials by an alternative, facile, ‘green’ route, including the production of novel bionanomaterials (45, 47, 48).
The aim of this work is to characterise the highly seleniferous soil (22) located in County Meath, Ireland, and to examine the relationship between soil geochemistry and the incumbent microbial community. This will provide an understanding of the geomicrobiological controls on Se stability and mobility in these environments and an understanding of the potential fate of agricultural Se amendments. Furthermore, the identification of naturally Se-tolerant microbial communities has potential for the development of novel bioremediation and biomineralisation strategies.

2. Materials and Methods

All chemicals used in this work were of analytical grade and supplied by Sigma Aldrich, UK, unless otherwise stated.

2.1 Field Site and Sampling Techniques

The field site was identified using data from Teagasc (19) and published literature, which had revealed an area near Trim, Co. Meath as having recorded soil Se concentrations exceeding 1200ppm at a depth of 15-30cm, and reports of Se toxicity in cattle (9, 22). The topographic range in the area is in the order of 20 metres, and the site is at the base of a small incline within agricultural fields. Soil at the site is waterlogged, due to the low-lying topography, and it has recently been changed from pasture to arable use. Groundwater Eh and pH measurements were taken at the field site using a Mettler Toledo SevenGo SG2. Complete cores of soil profiles were collected using an Eijkelkamp auger. The cores were maintained in anaerobic conditions by immediate transfer from the stainless steel core barrel into core-shaped polypropylene containers under a nitrogen atmosphere and stored at 10°C until analysis. Complete aerobic cores for chemical and organic analysis were transferred to storage in aluminium foil. In the laboratory, the aerobic cores were divided into ~10cm lengths and split into two for either chemical or organic biomarker analysis. Samples of soil were frozen in liquid nitrogen to preserve Se species.
2.2 Soil core characterisation

For analysis, half of the aerobic soil cores were dried at 70°C and powdered. The mineral component of the soil cores was then examined by X-ray diffraction (XRD) using a Bruker D8 Advance with a Cu kα source (1.54Å). Aliquots of the soil powder were separated for Loss on Ignition (LOI) analysis to determine total C content of the soils. Aliquots were also used to make wax-mounted pellets for X-ray fluorescence (XRF) analysis using an Axios Pw4400.

For lipid biomarker analyses the soils were freeze-dried, ground and soxhlet extracted to obtain the total lipid extracts. These were fractionated into acid, apolar and polar fractions, using a combination of Bond-Elut® and Al₂O₃ column chromatography, derivatized and analysed using an Agilent 789A gas chromatograph coupled to an Agilent 5975C MSD mass spectrometer (see supplementary information for details).

X-ray absorption spectroscopy (XAS) was used to determine Se speciation in the soil cores, undertaken on the high brilliance X-ray spectroscopy beamline ID26 of the European Synchrotron Radiation Facility (ESRF), Grenoble, France. The storage ring was operated at a nominal 6 GeV with a current of 1-200mA and the beamline utilises Si <111> monochromator crystals to deliver a spectral energy range of 2.4-27keV, encompassing the Se K-edge at ~12.6keV. X-ray Absorption Near-Edge Structure (XANES) spectra were collected in fluorescence mode over the energy range 12.63 to 12.7keV. Standards measured were powdered red amorphous and trigonal elemental Se (Se⁰), iron selenide (FeSe₂, Se²⁺), selenomethionine (C₅H₁₁NO₂Se, Se²⁺), sodium selenite (Na₂SeO₃, Se⁴⁺) and sodium selenate (Na₂SeO₄, Se⁶⁺) (figure 1) which were all ground and diluted with boron nitride. Soils and frozen aliquots from microcosms (described below) were mounted anaerobically onto a multisample stage for XAS and measured using a liquid nitrogen cryostat, allowing for XAS determination of Se in both solid and liquid phases.

2.3 Microcosm Experiments

Microcosm experiments were undertaken to assess the role of microorganisms in the cycling of Se at the field site (figure 2). 1g of anaerobic soil from the
seleniferous horizon (29-53cm) was added to 30ml serum bottles under an N$_2$ atmosphere.

Figure 1. XANES profiles for (top to bottom) (i) the Se standards, (ii) Co. Meath soil core, (iii) microcosms experiments and (iv) Se$^{VI}$
amended microcosm experiments. Edge energies for Se$^0$ (12654.0eV) and Se$^{VI}$ (12661.5eV) are highlighted (dashed lines).

Figure 2. Microcosm experiments were undertaken in 50ml serum bottles sealed with butyl rubber stoppers.

The composition of the groundwater at the field site was determined by ion chromatography (IC) using a Dionex DX600 and synthetic anaerobic groundwater of the same composition (29.8mM sodium bicarbonate, 1.34mM potassium chloride and 0.83mM magnesium sulphate) was added to the serum bottles to give a total volume of 10ml. Amendments to the groundwater were made to assess their effect on the mobility of Se.

The microcosms were incubated at 20°C under the following conditions; microaerophilic (the rubber stoppers were penetrated with needles to provide a constant air supply and the headspaces were replaced with 2 volumes of filter-sterilised air every 2 days), anaerobic, anaerobic plus sodium acetate (10mM), anaerobic plus sodium acetate (10mM) and sodium nitrate (10mM). The microcosms were duplicated and amended with sodium selenate (5mM). Prior to sampling, the bottles were shaken and samples of the soil suspension were taken at 7 day intervals with aliquots flash frozen in liquid N$_2$ and stored at -80°C for XAS. A second aliquot (1ml) was removed, centrifuged (13000 rpm, 5 mins) and the supernatants analysed by IC with a Dionex DX600 and ICP-AES with a Perkin Elmer Optima 5300. A third aliquot (0.5ml) was removed and digested in 0.5M HCl (4.5ml) for 1 hour for ferrozine analysis to determine
ferrous iron, followed by subsequent digestion with 200 µL hydroxylamine (6.26 N) for 1 hr and ferrozine analysis to determine total iron (39). Microcosm experiments were repeated in triplicate, and autoclaved controls for each condition tested were prepared and analysed.

2.4 Microbial Ecology

Aliquots of the microcosms were taken at each sample point for molecular ecology analysis. Samples were removed using sterile anaerobic microbiological techniques and flash frozen in liquid N₂ in 1.5ml eppendorf tubes prior to storing at -80°C. DNA was extracted from 200µl sediment slurry using a PowerSoil DNA Isolation Kit (MO BIO Laboratories INC, Solana Beach, CA, USA) following manufacturer suggested protocols. Total genomic DNA product for each sample was stored frozen at -20°C prior to PCR amplification. A fragment of the 16S rRNA gene approximately 1490bp in length was amplified from genomic DNA extract using broad-specificity primers 8F (14) and 1492R (34) using a BioRad iCycler (BioRad, Hemel Hempstead, Herts, UK). Purity of the amplified products was determined by electrophoresis in Tris-acetate-EDTA (TAE) gel. DNA was stained with ethidium bromide and viewed under short-wave UV light using a BioRad Geldoc 2000 system (BioRad, Hemel Hempstead, Herts, UK).

The 16S rRNA gene region of each sample was amplified in triplicate using the primers 8F (14) labelled with 6-FAM (6-carboxyfluorescein, Eurofins MWG Operon, London, UK); and 1492R (34) labelled with HEX (Hexachlorofluorescein, Eurofins MWG Operon, London, UK). PCR products were purified using the MinElute PCR purification kit (Qiagen, Crawley, UK). 150ng/µl of sample DNA was used for restriction digestion with enzymes HhaI and MspI (New England Biolabs, Inc.) in separate reactions using manufacturer recommended protocols. Size determination of T-RFs was performed using a LIZ 1200 size standard (Perkin Elmer Applied Biosystems, Warrington, UK) and an ABI Prism 3100 Genetic Analyser and Peak Scanner software (Applied Biosystems). Peaks recorded at less than 50bp in length were discarded. Duplicate data outputs allowed for the alignment of T-RF peaks and removal of erroneous data using T-
Align (58), assuming a standard confidence interval of 0.5bp. Comparison results files generated by T-Align for each enzyme digest were then transferred into PAST (28) statistical software for multivariate cluster analysis judging compositional dissimilarity between sample sites using the Bray-Curtis dissimilarity measure.

3. Results and discussion

3.1 Se in the soil components

Examination of the core revealed the soil to comprise a top layer of fine grained brown soil to a depth of 29 cm, below which was a ~13cm band of loosely consolidated, dry, dark brown/black soil, comprised of degraded plant fragments. Underlying this was a series of interbedded layers of brown soil and grey-blue clay (figure 3). XRD analysis of this soil core shows quartz as the dominant mineral throughout the core with minor amounts of calcite, illite and albite. A soil profile taken upslope from the boggy area (3m elevation) revealed a thinner, well drained soil (~70cm deep) with a disturbed top layer, no high organic layer and a progression into a more clay-rich lower profile.

XRF results of the soil core (table 1; figure 3) show that Se is enriched, with respect to the average global soil value of less than 2ppm, throughout the profile, with values ranging from 14.5ppm to 156.2ppm. The highest Se concentrations occur between 29cm and 53cm, peaking in the 29-43cm interval (figure 3). Several strong correlations with Se are observed in the XRF analyses, including positive correlations with total C, S and a range of toxic heavy metals (table 2). No correlation is seen between Se and Mo (table 2), despite high Se and Mo concentrations in the suggested source rock (46). Negative correlations are observed with common rock forming elements Si, Al and K (table 2). Ca and Mg are noted to be depleted in the seleniferous horizon (table 1), indicating that total C is likely organic rather than carbonate in origin.

Analysis of the second core taken slightly uphill from the first displayed much lower Se (3.4ppm max) and total C (6.7%wt) concentrations.
Figure 1 shows the characteristic absorption edge energies for a range of Se-containing standards, which increase with oxidation state (33); 12.6535keV and 12.6540keV for the reduced Se phases Se$^{II}$ and Se$^{0}$, respectively, 12.6555keV for organic phases such as selenomethionine, 12.6584keV for Se$^{IV}$ and 12.6615keV for Se$^{VI}$. The Se K-edge XANES for the soil cores (figure 1) show differences in the Se absorption edge energy and in post edge structure demonstrating that the Se species varies through the soil profile. The Se species in the top of the soil profile (0-10cm depth, 38.3ppm Se) shows an absorption edge at 12654.1eV, similar to the edge energy of inorganic Se$^{0}$ (figure 1), although differences in the post edge structure to the Se$^{0}$ standard indicates a reduced, organic Se phase is present. The XANES suggest the Se is present in the lower part of the profile as a form similar to that found at the surface (figure 1). Analysis of the highly seleniferous horizon (156ppm Se at 29-43cm depth) shows an absorption edge energy of 12655.2eV, 1.1eV higher in energy than the top and bottom horizons. This absorption energy closely matches that seen for Se bonded to methyl groups in the reduced organic Se phase selenomethionine (figure 1), although differences in the post-edge structure are visible.

<table>
<thead>
<tr>
<th>Depth (cm)</th>
<th>C (%)</th>
<th>Fe (%)</th>
<th>Ca (%)</th>
<th>S (%)</th>
<th>Si (%)</th>
<th>Se (ppm)</th>
<th>Cu (ppm)</th>
<th>Cd (ppm)</th>
<th>Pb (ppm)</th>
<th>U (ppm)</th>
</tr>
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<tr>
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<td><strong>2.8</strong></td>
<td><strong>5.3</strong></td>
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<td><strong>60</strong></td>
<td><strong>38.2</strong></td>
<td><strong>11.8</strong></td>
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<td>4.2</td>
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<td>5.2</td>
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<td>59.3</td>
<td>27.8</td>
<td>45.5</td>
<td>10.9</td>
<td>22.8</td>
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<td>61.8</td>
<td>14.5</td>
<td>26.5</td>
<td>4.2</td>
<td>21</td>
<td>3.4</td>
</tr>
</tbody>
</table>

*Table 1.* Chemical composition of the seleniferous soil core profile showing key elements.
Figure 3. (left) Variations in the Se, C, Si and lipid biomarkers throughout the soil core. (right) highlighted examples of regions within the soil cores. Dashed lines represent suggested boundaries of the horizons.
The strong correlation between Se and total C in the soil core profile indicates an association of Se with organic matter, and so organic biomarker analysis was undertaken. The lipid composition was analysed by GC-MS and both low molecular weight (LMW, <C$_{20}$) and high molecular weight (HMW, >C$_{20}$) straight chain n-alkanes, n-alkanoic acids and n-alkanols were present in high concentrations of up to 47 μg g$^{-1}$ (figure 3). All HMW distribution patterns show a clear odd-over-even (n-alkanes) or even-over-odd (n-alkanoic acids and n-alkanols) carbon chain predominance, consistent with a higher plant origin (15, 16). This is clearly reflected in the carbon preference indexes (CPIs) of all distribution patterns (table 3). Strong correlations exist between the Se concentrations and the amounts of HMW n-alkanoic acids, LMW n-alkanoic acids and LMW n-alkanols (table 2). In contrast no or weak correlation could be observed with the HMW n-alkanes and alkanols (table 2).

The XRF and organic analyses define three distinct geochemical horizons (figure 3); (i) an upper-most horizon extending from the surface to 29cm depth, consistent with recent agricultural disturbance and mixing of the O and A horizons; (ii) an organic-rich, peaty soil between 29 and 53cm depth with an extensive component of higher plant matter that is shown to contain elevated C, Se and heavy metal concentrations and; (iii) a lower, clay-rich layer extending below 53cm, indicating the presence of a small lake deposit as proposed by Parle and Fleming (46) and providing an impermeable base to the sequence. This is consistent with the topography of the site, with the low-lying area serving to drain the surroundings and slowly becoming infilled. The lowest layer analysed (70–75cm depth) displayed the lowest Se concentration of 14.5ppm, still considerably higher than the 0.1 – 2ppm global average Se soil concentration (4, 27). The dominance of quartz and limited carbonate throughout the seleniferous soil profile alongside the presence of an impermeable clay base suggests the soil has been developed on reworked sediment, rather than directly on the interbedded limestones and mudstones of the underlying Lucan Formation. This is further supported by the decreasing Se
concentration with increasing depth below the reworked horizon, a trend opposite that expected if the Se was originating from the underlying bedrock. High levels of Se (156.2ppm) are found within a narrow, carbon-rich (33.8wt% total C) horizon between 29 and 43cm depth. This band also contains elevated concentrations of heavy metals (table 1) and HMW and LMW n-alkanes, n-alkanoic acids and n-alkanols (figure 3), indicative of significant contributions of higher plant and bacterial matter.

The Se is strongly coordinated throughout the soil profile with total C ($R^2$=0.96), and XANES analysis precludes the presence of inorganic Se (figure 1). The absorption edge energy and structure are similar to that seen for the organic Se phase selenomethionine, and published XANES results of Ryser et al (56) for a number of organoselenium compounds show how the edge energy and structure can vary between otherwise very similar compounds, owing to the degree of covalency in Se$^{II}$ phases. It can however be deduced that the Se within this horizon is likely found as a reduced organoselenium form similar to selenomethionine.

Further characterisation of the organic phases by GC-MS showed positive correlations with Se for HMW and LMW n-alkanoic acids and LMW n-alkanols (table 2), although the strength of these correlations were notably lower than that seen for total C.

The field, XRF, organic and XANES findings are consistent with the concentration of Se in a small, low-lying fen, which formed as a result of the infilling of a pre-existing postglacial lake which served to drain the surrounding area directly overlying seleniferous components of the Lucan Formation, as proposed by Fleming et al (22, 42, 46). This hypothesis is supported by the much lower Se concentrations reported in the soil core taken uphill, suggesting downhill movement of percolating, Se-bearing drainage waters. The strong correlation between total C and Se, stronger than any individual organic form tested (table 2), and the large higher plant content indicated both by lipid biomarker analysis and observations of plant material within the seleniferous horizon suggests phytoconcentration was the mechanism by which Se was able to accumulate. The lipid biomarkers are dominated by $C_{27}$, $C_{29}$ and $C_{31}$, consistent with
significant terrestrial higher plant input, and likely originating from the epicuticular waxes of vascular plants (64, 71).

<table>
<thead>
<tr>
<th>Correlations with Se</th>
<th>$R^2$</th>
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**Inorganic Components**

<table>
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<th>Element</th>
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<tbody>
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</tr>
<tr>
<td>Al</td>
<td>0.79 (negative)</td>
</tr>
<tr>
<td>K</td>
<td>0.76 (negative)</td>
</tr>
<tr>
<td>S</td>
<td>0.97</td>
</tr>
<tr>
<td>Cu</td>
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<td>Cd</td>
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</tr>
<tr>
<td>U</td>
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<tr>
<td>Pb</td>
<td>0.83</td>
</tr>
<tr>
<td>Mo</td>
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</tr>
</tbody>
</table>

| Total C | 0.96 |

**Organic Biomarkers**

<table>
<thead>
<tr>
<th>Biomarker Type</th>
<th>$R^2$</th>
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<tbody>
<tr>
<td>HMW n-alkanes</td>
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<tr>
<td>HMW n-alkanoic acids</td>
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<td>HMW n-alkanols</td>
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<td>LMW n-alkanoic acids</td>
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<td>LMW n-alkanols</td>
<td>0.81</td>
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*Table 2.* Correlations ($R^2$) values for a range of soil chemicals and organic components.

The increase in the $C_{23}$:$C_{29}$ ratio (table 3), the medium molecular weight lipid biomarkers typically associated with *Sphagnum* species vs. the higher molecular
weight lipids of terrestrial vascular plants (44, 71), indicates that macrophytic plants were more prevalent during the deposition of the seleniferous horizon. Calculated $P_{AQ}$ concurs with these findings; values are consistently between 0.1 and 0.4, indicative of emergent macrophytes (table 3) (21, 44, 71). The lipid biomarkers also allow for a relative palaeoclimatic reconstruction at the time of deposition based upon the varying ratios of the organic materials. Elevated $P_{AQ}$ values and a noticeable increase in the ratios of the $C_{23}:C_{25}$ and the $C_{23}:C_{29}$ $n$-alkanes (table 3) are indicative of a wetter climate at the time of deposition of the seleniferous horizon relative to the horizons above and below (21, 44, 64, 71). There is also an appreciable decrease in the $n$-alkane average chain length (ACL) from the lower horizon 3 in to the seleniferous horizon 2, suggestive of a transition to a cooler climate during the deposition of the seleniferous organics. Rapid deposition and burial under these conditions would serve to preserve the Se-enriched plant matter as the peat-like material found, hindering microbial degradation and the release of Se.

Uptake and enrichment of Se by higher plants has been previously noted; the localisation within the plant body is largely dependent on plant species, with Se possibly located within leaves, stems, rhizomes and/or roots as a range organic Se compounds, Se-amino acids, selenoproteins and their biochemical precursors including Se-methylSec, methylselenol, selenocysteine and selenomethionine (1, 2, 6, 12, 38, 49).

Variations seen in the XANES profile of the soil core indicates that Se is found as a distinct form within the seleniferous horizon compared to that above and below (figure 1), and represents upper and lower boundaries for Se phytoconcentration within the site. It is also likely that recent reworking has lead to a decrease in the topsoil Se concentration from the 360ppm-1200ppm (0-15cm and 15-30cm depth, respectively) concentration reported by Fleming (22).

The role of bacteria in the concentration of Se at this site can also be examined. As noted, positive correlations exist between the LMW (likely bacterial) lipids and Se (table 2), and analysis of the $n$-alkanol biomarkers infers a significant input of LMW organics at the seleniferous horizon (figure 3). These findings
contradict those reported for the \( n \)-alkanes, which suggest higher plant materials dominate. High proportions of \( C_{16} \) \( n \)-alkanoic acids are found within the soil profile, consistent with microbial reworking of the organic sediments (71). Published literature (8) note that \( n \)-alkanols and \( n \)-alkanoic acids are amongst the least recalcitrant lipids to bacterial degradation, and it is therefore likely the correlation seen between the LMW components and the Se is owing to increased levels of bacterial activity within the seleniferous horizon, owing to the presence of substantial amounts of organic material. There is no indication that the increased Se concentration is directly attributable to the microbial activity within this layer.

<table>
<thead>
<tr>
<th>Depth (cm)</th>
<th>CPI(^A)</th>
<th>CPI</th>
<th>CPI</th>
<th>( C_{23}:C_{25} ) ( n )-alkane</th>
<th>( C_{23}:C_{29} ) ( n )-alkane</th>
<th>( P_{aq} )^C</th>
<th>ACL(^D)</th>
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</thead>
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<tr>
<td>Alkanes</td>
<td>FA(^B)</td>
<td>Alkanols</td>
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<tr>
<td>62-70</td>
<td>6.1</td>
<td>0.27</td>
<td>28</td>
<td>0.48</td>
<td>0.17</td>
<td>0.17</td>
<td>29.5</td>
</tr>
</tbody>
</table>

*Table 3. Lipid biomarker variations throughout the soil profile.\(^A\)*

To express the \( n \)-alkane odd-over-even predominance of the high molecular weight (HMW) hydrocarbons, carbon preference index (CPI) is used which can be calculated using the following equation (63): 
\[ \text{CPI} = \frac{\frac{1}{2} \sum (X_i + X_{i+2} + … + X_n) / \sum (X_{i+1} + X_{i+3} + … + X_{n-1}) + \frac{1}{2} \sum (X_i + X_{i+2} + … + X_n) / \sum (X_{i+1} + X_{i+3} + … + X_{n-1})}{\sum (X_{i+1} + X_{i+3} + … + X_{n-1})} \]

\(^B\)Fatty acids, \( n \)-alkanoic acids; \(^C\)\( P_{aq} = (C_{23} + C_{25}) / (C_{23} + C_{25} + C_{29} + C_{31}) \) (21, 44, 71); \(^D\)Average Chain Length 

\[ \text{ACL} = \frac{[25(nC_{25}) + 27(nC_{27}) + 29(nC_{29}) + 31(nC_{31}) + 33(nC_{33})] / [nC_{25} + nC_{27} + nC_{29} + nC_{31} + nC_{33}]} \]
3.2 Microcosm Experiments

Microcosm experiments using soil from the highly seleniferous horizon (29-53cm) were set up to investigate the role of microbial communities in the cycling and mobility of selenium in natural soils with very high selenium concentrations.

3.2.1 Microcosms without additional Se.
Significant Fe$^{II}$ was observed in microcosm solutions after 1 week of incubation at 20°C in all of the conditions tested, with the exception of sterilised controls (figure 4). High initial rates of Fe$^{III}$ reduction occurred in anaerobic microcosms, with extractable Fe$^{II}$ concentrations of ~30mM after 4 weeks incubation. Fe$^{III}$ reduction rates were comparable in soils both with and without additional electron donor (sodium acetate, 10mM). The addition of 10mM nitrate to the microcosms resulted in rapid denitrification, with all nitrate removed in less than 1 week (figure 4). A decrease in the rate and extent of Fe$^{III}$ reduction was observed with the addition of 10mM nitrate. The microcosms were sampled at weeks 0, 2 and 4 for XAS analyses (figure 1), and show the Se k-edge absorption energy at 12655.2eV did not change throughout the course of the experiment. The onset of Fe$^{III}$ reduction in the microcosms is caused by microbial Fe metabolism under reducing conditions, and the high organic content of the samples was found to be sufficient to support the microbial community as the addition of 10mM acetate had little impact. The simultaneous addition of nitrate to the microcosms provided an alternative electron acceptor, thus decreasing the rate of Fe$^{III}$ reduction. Detectable Fe$^{II}$ in solution in the microaerophilic microcosms suggested that a high level of microbial activity, coupled with a high concentration of organic material, led to depleted oxygen levels within these microcosms and reduction of Fe$^{III}$ by anaerobic microbial metabolism.
Despite the high levels of microbial activity recorded in these soils, under both aerobic and anaerobic conditions, the reduced organic Se phase did not change or enter the mobile aqueous phase indicating that the organic Se phase is recalcitrant to microbial degradation within this time frame.

3.2.2 SeVI-amended microcosms
Microcosms were amended with 5mM SeVI to assess the cycling of bioavailable Se oxyanions in naturally seleniferous soils. No FeIII reduction or SeVI reduction was observed in the autoclaved control microcosms, indicating that all changes measured in the test microcosms were a result of microbial activity. As with the un-amended microcosms, low concentrations of FeII were measured in solution in the solids incubated under microaerophilic conditions, indicating that microbial activity, even in the presence of high SeVI concentrations, was sufficient to deplete the oxygen levels to the extent where FeIII reduction could
take place (figure 5). Reduction of Se$^{VI}$ was also measured in the microaerophilic microcosms at a rate of 50µM day$^{-1}$ with 67% remaining in solution after four weeks. The reduction of Se$^{VI}$ proceeded via Se$^{IV}$, with 0.5mM measurable in solution after four weeks (figure 5). The rate of Se reduction was much faster in the anaerobic microcosms with 100% of the Se$^{VI}$ removed from solution within 1 week. Se$^{IV}$ (0.8mM) was measured in solution after 1 week at 20°C, but was completely removed from solution after 2 weeks. Reduction of Fe$^{III}$ also occurred under anaerobic conditions in the presence of Se$^{VI}$, with a steady increase in extractable Fe$^{II}$ of 52µM hr$^{-1}$ over the 4 week period. The initial Fe$^{III}$ reduction rate in the presence of Se$^{VI}$ was half that measured in the un-amended microcosms, but the total extractable Fe$^{II}$ was the same both in the presence and absence of Se$^{VI}$ after incubation for four weeks. No significant change in the rate of Fe$^{III}$ or Se$^{VI}$ reduction upon addition of acetate (10mM) was observed in the Se$^{VI}$ amended microcosms, confirming that the organic-rich, seleniferous soil horizons were not limited by a lack of electron donors. Rapid denitrification occurred in the Se$^{VI}$-amended microcosms with acetate and nitrate, and all nitrate was removed from solution within 1 week. In these Se amended microcosms, the rate of Fe$^{III}$ reduction in the presence of nitrate was reduced by 27% relative to microcosms without nitrate. All amended Se was removed from solution after 3 weeks incubation, rather than after 2 weeks in the absence of 10mM nitrate. Both Se$^{VI}$ (0.9mM) and Se$^{IV}$ (2.4mM were measurable after 1 week and Se$^{IV}$ (0.4mM) was still measurable after 2 weeks.

The Se amended microcosms were sampled at weeks 0, 2 and 4 for XAS analyses (figure 1). The XANES of the week 0 microcosms are consistent with the presence of Se$^{VI}$ and the decrease in absorption edge energy (of 7.5eV) in the anaerobically incubated microcosms.
Figure 5. Geochemical data for Se\textsuperscript{VI} amended microcosms. (A) microaerophilic microcosms, (B) anaerobic microcosms, (C) anaerobic and acetate, (D) anaerobic, acetate and nitrate. Key to symbols: (solid squares) Fe\textsuperscript{II}, (open circles) Se\textsuperscript{VI}, (open triangles) Se\textsuperscript{IV}, (open squares) NO\textsubscript{3}\textsuperscript{−}.

Comparison of the XANES of the Se\textsuperscript{VI}-amended microcosms with those for a range of Se standards (figure 1) shows that the Se was reduced from Se\textsuperscript{VI} (edge energy 12661.5eV) to insoluble Se\textsuperscript{0} (12654.0eV) by week 2. No intermediate Se\textsuperscript{IV} phase was detected in the XANES. XANES for the microaerophilic microcosms showed increasing proportions of Se\textsuperscript{IV} and Se\textsuperscript{0} with a decreasing Se\textsuperscript{VI} intensity over the 4 week period, as Se reduction took place. After 4 weeks incubation under microaerophilic conditions, the XANES shows no evidence for the presence of Se\textsuperscript{VI}, however 2.9 mM Se\textsuperscript{VI} was detected by IC, highlighting the necessity for complementary analytical techniques for complex environmental samples containing both solid and aqueous phases.
The Se concentration of the amended microcosms was also sufficient to collect EXAFS data. Theoretical fitting of the EXAFS oscillations and resultant Fourier transforms (figure 6) indicate an inner shell of two Se scatterers at 2.37Å; with a second outer shell of two Se atoms at 3.33Å. These findings are consistent with red, elemental α-Se (48) and confirm the presence of this phase in the microcosms.

![Figure 6](image)

*Figure 6.* Se EXAFS and Fourier transform for the Anaerobic + Se sample. Empirical data (solid lines), model fit data (dashed lines).

### 3.2.3 Microbial community analysis

Bray Curtis similarity measures (figure 7) show that the microbial community remained largely unchanged after four weeks incubation at 20°C under anaerobic conditions, indicating the seleniferous, organic-rich horizon contains an anaerobic microbial community. The T-RFLP results for the initial microbial community and for the community after four weeks incubation with or without 10mM nitrate remain 60-65% similar, inferring that the original community was composed largely of nitrate reducing bacteria. These findings are concordant with the agricultural use of the land, where nitrate supplementation through fertilisation is common. Major changes in community can be seen with the addition of 5mM SeVI to the soils, with only 15% and 28% similarity (*Msp I* and *Hha I*, respectively) between anaerobically incubated soils with and without SeVI. The natural microbial community is also affected by the combined addition of nitrate and SeVI, as, after 4 weeks of incubation, the community is only 35-
45% similar to Se$^{VI}$ amended anaerobic microcosms without nitrate and 15-28% similar to nitrate amended microcosms without Se$^{VI}$.

Figure 7. (Left) NMDS plot for the TRFLP data obtained from the Co. Meath microcosms using the 8F-FAM primer and Hha I restriction enzyme. (Cross) microcosm start, (open circle) microcosm 4 weeks, (solid circle) microcosm 4 weeks with Se$^{VI}$, (open square) microcosm 4 weeks with nitrate, (solid square) microcosm 4 weeks with nitrate and Se. (Right) Dendrogram of TRFLP results using 8F-FAM primer and Hha I restriction enzyme.

These findings indicate that 5mM Se$^{VI}$ is toxic to much of the naturally abundant nitrate reducing community. However, the microbial community does contain Se-resistant organisms that are able to drive redox reactions resulting in the reduction of Se$^{VI}$ to Se$^{0}$. The enrichment of these Se-resistant organisms is observed in the large change in microbial community in the presence of Se$^{VI}$, as opposed to the lack of change in the un-amended microcosms.

Under the range of geochemical and microbial conditions tested in this study, the high concentration of Se naturally present in the soil at this field site does not become bioavailable through release into the aqueous environment, but remains in the form of reduced organic Se associated with the solid phase. Upon amendment with 5mM Se$^{VI}$, rapid microbially-driven reduction occurs
and, under anaerobic conditions, Se is completely removed from the aqueous phase within one week, with Se⁰ as the dominant reduced Se phase at the end of 4 weeks incubation at 20°C. The presence of alternative oxidants, such as oxygen or nitrate, has an inhibitory effect on the microbially-driven reduction of both Fe³⁺ and Se⁶⁺. The diminished rate of Fe³⁺ and Se⁶⁺ reduction in nitrate amended microcosms can be attributed to the large shift in microbial community, however several contributory chemical and biological factors could also explain this reduction in activity; (i) direct competition between nitrate and selenate for complexation with nitrate reductases due to the higher affinity of nitrate (57); (ii) in aqueous systems, the redox potential of the NO₃⁻/NO₂⁻ couple (+0.42V) is similar to that of SeO₄²⁻/SeO₃²⁻ (+0.44V) and so can directly compete with Se⁶⁺ for reducing equivalents, inhibiting formation of an insoluble Se⁰ phase (41, 59, 66, 70); (iii) the reoxidation of reduced Se phases by nitrate has been demonstrated and implicated in selenium toxicity reports in the western United States (67). Comparison between un-amended microcosms and microcosms amended with 5mM Se⁶⁺, incubated under anaerobic conditions, shows a 59% decrease in the initial rate of Fe³⁺ reduction as determined by extractable Fe⁰ concentration (128μM hr⁻¹ vs 52μM hr⁻¹), but the extent of Fe³⁺ reduction is similar over the 4 week incubation period, as evidenced by the similar concentrations of Fe⁰ in solution (figure 4; figure 5). Murphy (43) shows that Fe⁰ hydroxides are capable of the reduction of Se⁶⁺ to amorphous, red α-Se⁰ at greater than circumneutral pHs via the reaction shown by the equation below.

\[
\text{Na}_2\text{SeO}_4 + 9\text{Fe}^\text{II}(\text{OH})_2 \rightarrow \text{Se}^\text{0} + 3\text{Fe}_3\text{O}_4 + 2\text{NaOH} + 8\text{H}_2\text{O}
\]

The complete reduction of 5mM Se⁶⁺ by Fe⁰ hydroxides would result in the release of 10mM NaOH into the microcosms. However, a significant change in pH was not observed in the microcosms amended with Se⁶⁺, with a small increase of 7.7 to ~8 measured in all microcosms, including sterile controls. This lack of significant change in the pH coupled with the T-RFLP data showing a large community change indicate that the lower initial concentrations of Fe⁰ in solution are due to a lower initial Fe³⁺ reduction rate, rather than chemical
reduction of $\text{Se}^{\text{VI}}$ by the $\text{Fe}^{\text{II}}$. It is therefore suggested that $\text{Se}^{\text{VI}}$ reduction measured in the microcosms is a result of direct microbial reduction.

4. Conclusion

This research has identified and examined the geochemical and geomicrobiological characteristics of a naturally seleniferous soil located in Co. Meath, Ireland. Strong positive correlations have been noted for Se with a range of toxic heavy metals, and principally with the total carbon content. The field site in Co. Meath remains highly contaminated with Se, with one 14cm interval containing 156ppm, much higher than the global average of 0.1-2ppm (4, 27). The source of the Se in the area is attributed to the local seleniferous layers within the Lucan Formation (22, 42, 46), and the form of the Se is indicated to be as a reduced organic phase within plant material. Accumulation and enrichment of Se within plants has been previously shown (1, 2, 6, 11, 37, 49), and the preservation of organic matter within a fen has prevented the release of Se back into the environment by microbial degradation, thus providing a sink for Se.

Active bacterial communities have been demonstrated within the seleniferous horizon, both by microcosm experiments and by lipid biomarker proxies, and the rapid removal of amended nitrate with little change in microbial community structure indicates that nitrate reducing bacteria are prevalent. The strong correlation between bacterial biomarkers and Se concentration is likely a function of the higher microbial activity in areas of abundant carbon sources, rather than an indication of a bacterial Se concentration mechanism. Despite the active bacterial communities shown during microcosm experiments, no Se was remobilised to concentrations above detection limits on the time scale tested, suggesting the organic Se was recalcitrant to microbial degradation.

Amendment of the soils with $\text{Se}^{\text{VI}}$ saw rapid, microbially-driven Se reduction producing the immobile, red element Se phase; a less toxic form which displays a lower bioavailability, with important implications for the efficacy of $\text{Se}^{\text{VI}}$ addition in agricultural areas. The addition of nitrate hinders $\text{Se}^{\text{VI}}$ reduction,
with resulting large shifts in the bacterial community structure suggesting the incumbent nitrate reducing community are largely intolerant to Se\textsuperscript{VI}. These findings indicate that the addition of Se\textsuperscript{VI} as a component of nitrogenous agricultural fertilisers may lead to a decrease in local Se immobilisation, decreasing effectiveness as a fertiliser and increasing Se in surface run-off. It has been demonstrated that the bioavailability (and therefore the chemical form) of Se in these environments plays a crucial role in determining the incumbent microbial community. At this field site, the Se present as a reduced organic phase is not bioavailable. The amendment of the seleniferous soils with toxic concentrations of bioavailable Se\textsuperscript{VI} resulted in a large reduction in the diversity of the naturally-occurring microbial population. Further research is required into the microbes that survive and play a role in the reduction of the toxic bioavailable Se to find novel, environmentally friendly bioremediation strategies and biomineralisation techniques for the production of commercially relevant Se-bearing nanophases (36, 37, 45, 47, 48).

**Acknowledgments**

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Supplementary Information

Additional information on Materials and Methods

Organic lipid analyses

For organic lipid analyses, soils were freeze-dried, ground and 15-20g was extracted using a Soxhlet apparatus with a dichloromethane (DCM)/methanol (MeOH; 2:1 (v/v)) mixture for 24 hrs. The total lipid extracts (TLEs) obtained were concentrated using rotary evaporation, aliquots were taken and a mixture of standards (5.4 μg of tetracosane-d₅₀; and 10.8μg of 2-hexadecanol) were added. Subsequently, the aliquots were separated into two fractions using Bond-Elut® column chromatography (Strata NH₂; 500mg, 6mL, glass; ISOLUTE; eluting with DCM/isopropanol (2:1, v/v; 12mL; ‘neutral lipid fraction’) and 2% acetic acid solution in diethyl ether (12mL; ‘acid fraction’). The neutral lipid fractions were further separated into two fractions using a column packed with (activated) Al₂O₃ by eluting with hexane/DCM (9:1 v/v; 3mL; “apolar fraction”) and DCM/MeOH (1:1 v/v; 3mL; “polar fraction”). The polar fractions were dissolved in bis(trimethylsilyl)trifluoroacetamide (BSTFA; ALDRICH), and heated (70°C; 60 min) to convert alkanols into their trimethylsilyl ethers. The acid fractions were derivatized, after addition of 5.40μg of tetracosane-d₅₀ standard, with bromotrifluoride (BF₃) in MeOH and heated (70°C; 60 min) to convert the acids into their corresponding methyl esters. Blanks were run to ensure that no contamination was introduced during the extraction and separation procedures and were found to be less than 1:100 for all target analytes. All fractions were analysed using gas chromatography mass spectrometry (GC-MS) using an Agilent 789A GC interfaced to an Agilent 5975C MSD mass spectrometer operating with electron ionization at 40eV and scanning from m/z 50-600 at 2.7 scans/sec. The GC was equipped with an Agilent 7683B auto sampler and a programmable temperature variation (PTV) inlet. The samples were dissolved in hexane prior to injection, injected using a pulsed spit-less injection (1µL; inlet pressure of 25 psi for 0.25 min) and separated on HP-5 capillary column (J & W scientific column 5% diphenyldimethyl polyseloxcane; length 30m, I.D. 320μm, film thickness 0.25μm). The samples were run at constant flow (1mL min⁻¹) with
helium as a carrier gas. The heated interface, the mass source and the MS quadrapole temperatures were set to 280°C, 230°C and 150°C, respectively. The samples were injected at 70°C and the oven was programmed to 130°C at 20°C min⁻¹ and then at 4°C min⁻¹ to 300°C at which it was held isothermally for 25 minutes. The quantitative data were obtained by comparing the individual peak areas with a known concentration of the internal standard used.

Additional information on Results

*Figure 1.* (Left) NMDS plot for the TRFLP data obtained from the Co. Meath microcosms using the 1492R-HEX primer and *Hha I* restriction enzyme. (Cross) microcosm start, (open circle) microcosm 4 weeks, (solid circle) microcosm 4 weeks with Se⁶⁺, (open square) microcosm 4 weeks with nitrate, (solid square) microcosm 4 weeks with nitrate and Se. (Right) Dendrogram of TRFLP results using 1492R-HEX primer and *Hha I* restriction enzyme.
Figure 2. (Left) NMDS plot for the TRFLP data obtained from the Co. Meath microcosms using the 8F-FAM primer and Msp I restriction enzyme. (Cross) microcosm start, (open circle) microcosm 4 weeks, (solid circle) microcosm 4 weeks with Se\textsuperscript{VI}, (open square) microcosm 4 weeks with nitrate, (solid square) microcosm 4 weeks with nitrate and Se. (Right) Dendrogram of TRFLP results using 8F-FAM primer and Msp I restriction enzyme.

Figure 3. (Left) NMDS plot for the TRFLP data obtained from the Co. Meath microcosms using the 1492R-HEX primer and Msp I restriction enzyme. (Cross) microcosm start, (open circle) microcosm 4 weeks, (solid circle) microcosm 4 weeks with Se\textsuperscript{VI}, (open square) microcosm 4 weeks with nitrate, (solid square)
microcosm 4 weeks with nitrate and Se. (Right) Dendrogram of TRFLP results using 1492R-HEX primer and \textit{Msp I} restriction enzyme.
Chapter 5

Research paper 2

Reductive biosynthesis of elemental selenium and tellurium nanoparticles by Geobacter sulfurreducens

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Reductive biosynthesis of elemental selenium and tellurium nanoparticles by

*Geobacter sulfurreducens*

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**Abstract**

*Geobacter sulfurreducens* can precipitate Se\(^0\) and Te\(^0\) from the dissimilatory reduction of Se\(^{IV}\) and Te\(^{IV}\) oxyanions, however the organism cannot conserve energy for growth from these reactions, and small concentrations of Se\(^{IV}\) and Te\(^{IV}\) oxyanions are highly toxic (MIC values \(~10\mu M\)). The precipitation of Te\(^0\) and Se\(^0\) nanomaterials under non-growth conditions is reported, and the ability to control the morphology and particle size is investigated. Te\(^0\) nanorods 50-200nm in length and clusters of Te\(^0\) nanospheres \(~20\text{nm}\) in diameter are reported, as well as a range of sizes and distributions of Se\(^0\) nanospheres, typically 100-200nm in diameter. Biogenic Se\(^0\) nanospheres are more stable over time than abiotic counterparts, the latter crystallising within 53 days, with biogenic Se\(^0\) remaining stable in aqueous suspension and as a dried powder for over a year. The formation of trigonal Se\(^0\) crystals is observed over time under alkaline conditions. Final particle sizes and size distributions of Se\(^0\) produced by *G. sulfurreducens* can be controlled by varying the initial incubation conditions, and extracellular bacterial proteins are implicated in controlling the particle stability, size and morphology.
Keywords: Geobacter sulfurreducens, tellurium, selenium, bionanomineralization, chalcogen.

1. Introduction

The chalcogens selenium and tellurium are found naturally in a number of oxidation states; they occur as the hexavalent oxyanions selenate and tellurate (\(\text{SeO}_4^{2-}/\text{TeO}_4^{2-}\)) and tetravalent selenite and tellurite (\(\text{SeO}_3^{2-}\) and \(\text{TeO}_3^{2-}\)), as well as in elemental form (\(\text{Se}^0, \text{Te}^0\)) and as the reduced anions selenide and telluride (\(\text{Se}^{2-}, \text{Te}^{2-}\)). In addition they can be present as a range of organic and biochemical compounds (51). The use of Se and Te in chemical and technological applications is widespread. Investigations into the use of \(\text{Te}^0\) in nanoelectronic and nanophotonic devices are ongoing (33) and metal telluride quantum dots offer high photostability, high quantum yields and tuneable emissions (4, 5, 16, 39). Selenium has unique physico-chemical properties which have resulted in technological applications such as in photoelectric cells, semiconductors and rectifiers. Chemical applications include catalysis, dye reduction, steel and ceramics manufacturing and sequestration of hazardous wastes (6, 19, 22, 25, 40, 49).

Continuing interest in nanomaterials has generated research into the formation of \(\text{Se}^0\) and \(\text{Te}^0\) nanowires and nanoparticles, both via chemical (2, 7, 10, 15, 18, 20, 26, 27, 32, 38, 47, 52) and biological routes (3-5, 13, 21, 24, 33, 41-44). In the latter case, biosynthesis of novel, technologically relevant nanomaterials promises economic and environmental advantages, utilising the unique biochemical reduction pathways offered by microorganisms to produce products with distinct properties compared to those produced by chemical means (4, 5, 21, 29, 33, 35, 36, 40, 42).

The formation of elemental Se and Te phases from soluble oxyanions by the Fe\(^{III}\)-reducing bacterium *Shewanella oneidensis* has been demonstrated and c-type cytochromes have been implicated in the reduction pathway (21, 24, 41, 44). Work by Abdelouas *et al* (1) has also shown that isolated suspensions of bacterially derived cytochrome \(c_3\) are capable of the formation of elemental
selenium nanowires. Direct comparison in the reduction of SeIV between S. oneidensis and another well characterised, FeIII-reducing subsurface bacterium Geobacter sulfurreducens has shown that the latter organism, which also contains abundant c-type cytochromes, was able to reduce SeIV at a higher rate (41).

The current research investigates the ability of G. sulfurreducens to utilise SeIV and TeIV to conserve energy for growth, coupling the oxidation of hydrogen or acetate to the reduction of these oxyanions. Non-growing or ‘resting’ cell cultures were investigated for their ability to produce significant quantities of technologically and industrially relevant Se0 and Te0 nanoparticles, and to determine whether it is possible to produce predetermined desired characteristics by altering the initial incubation conditions. The precipitates formed were characterised by a range of analytical techniques including ion chromatography, transmission and scanning electron microscopy (TEM/SEM) coupled with energy dispersive spectroscopy (EDX) and particle size determination using disc centrifugation.

2. **Experimental details**

All chemicals used in this study were of analytical grade and obtained from Sigma Aldrich (UK). Prior to experimentation, G. sulfurreducens PCA (ATCC 51573) was grown at 30°C under anaerobic conditions at pH7 for 24 hours in defined medium (34) amended with 20mM sodium acetate as the electron donor and 40mM fumaric acid as the electron acceptor.

2.1. **Growing cell experiments**

In order to test the ability of G. sulfurreducens to couple the reduction of SeIV and TeIV to growth in the absence of any alternative electron acceptor, a previously defined medium with 25mM sodium acetate (34) as the electron donor was amended with 1mM sodium selenite/tellurite as the sole electron acceptor. Cell enumeration was determined by epifluorescence microscopy.
using the cationic dye acridine orange, with stained cells mounted onto 0.2µm black membrane filters via vacuum filtration (37).

For determination of maximum inhibitory concentrations (MIC) of the metalloids, defined medium with 25mM acetate and 40mM fumarate (34, 41) under an N₂ atmosphere was amended using aseptic, anaerobic techniques with concentrations in the range of 0.01 to 10mM sodium tellurite or sodium selenite. Late log phase *G. sulfurreducens* cultures were inoculated (5 vol%) and incubated at 30°C, in the dark for 7 days. Cell numbers were determined by epifluorescence microscopy as described.

Ion chromatography (IC) analysis was used to measure Se⁴⁺ and Te⁴⁺ concentrations; Se⁴⁺ was determined via variations in conductivity using a Metrohm 761 IC fitted with a Dionex AS9-HC column coupled to a Dionex AG9-HC guard column and a 9mM sodium carbonate mobile phase. Te⁴⁺ was determined photometrically using a Camspec UV spectrophotometer at a wavelength of 220nm with a Dionex DX600 fitted with a Dionex AS9-HC column and using a 9mM sodium carbonate mobile phase.

2.2. Resting cell experiments

The ability of *G. sulfurreducens* to produce Se⁰ and Te⁰ nanoparticles under non-growth conditions was tested. *G. sulfurreducens* was grown to stationary phase in a defined medium (34) amended with 25mM sodium acetate and 40mM fumaric acid. Cells were isolated and washed by centrifugation (14) prior to resuspension in 20mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer at pH7 to an optical density (600nm) of 0.4. Cultures were then amended with sodium selenite (1mM) and sodium tellurite (1mM), along with 10µM of the electron shuttle anthraquinone-2,6-disulphonate (AQDS), and the headspaces were purged and replaced with H₂ to act as an electron donor. Cultures were then incubated at 30°C in the dark without agitation for 48 hours, upon which a red or black precipitate was visible with Se⁴⁺ and Te⁴⁺ respectively.

To investigate whether the size and/or morphology of Se⁰ precipitates could be varied for specific applications by altering the initial incubation conditions, a
matrix of experiments was designed to test the effects of cell concentration, growth phase of harvested cells, O₂ concentration, pH, electron donor (H₂ or acetate) and the effect of the addition of the redox mediator AQDS. Cells were grown to late log phase (16.5 hours growth) in defined media at 30°C in the dark and compared to those grown to stationary phase for 24 hours. *G. sulfurreducens* cells were harvested as described previously (14) and resuspended in appropriate buffer solutions. Cells were added to sterile, anaerobic solutions of the buffer 2-(N-morpholino)ethansulfonic acid (MES; 20mM) for experiments at pH5.5 (pKa ~6.2), 20mM MOPS buffer for experiments at pH7 (pKa ~7.2) and 20mM tricine buffer for experiments at pH8.5 (pKa ~8). Buffer solutions were then amended with sodium selenite (1mM). Cell concentrations for the experiments were 3.6 x10⁸ cells ml⁻¹. Where acetate was used as the electron donor, solutions were amended with sodium acetate (4mM); where hydrogen was used as the electron donor, the N₂ headspaces were flushed for 1 minute with pure H₂. For microaerophilic experiments, the butyl rubber septa were pierced with two sterile 0.8mm bore needles capped with 0.22µm sterile filters to prevent contamination. The effect of an electron shuttling compound was assessed with the addition of AQDS to a final concentration of 2 µM. To quantify the rate of Se⁴ reduction for this matrix of incubation conditions, aliquots were taken regularly over a 48 hour period and analysed for Se⁴ by IC. To characterise the affinity of whole cells for Se⁴, the rate of reduction from a known cell concentration was determined across a range of Se concentrations (0.01 to 10mM) and used to calculate the Michaelis constant (Kₘ) and the maximum reaction velocity (Vₘₐₓ) for the Se⁴ oxyanion.

2.3. **Nanoparticle characterisation**

The Se and Te precipitates formed under a range of conditions were characterised by scanning electron microscopy (SEM) using a Philips XL30 FEG-ESEM with elemental analysis by an EDAX Gemini EDX system. Samples were transferred directly onto carbon pads mounted on Al sample stubs to avoid the overlap of the Al K-edge emission (1.485keV) with the Se L-edge emission (1.381keV). Transmission electron microscopy (TEM) was performed on a Philips
CM-200 FEG-TEM operated at 200kV with elemental analysis by an Oxford Instruments ISIS EDX system. Selected area electron diffraction (SAED) was also performed to determine crystallinity. Sample aliquots were mounted directly on to carbon-coated holey grids (Agar Scientific, UK).

Freeze dried Se⁰ particles formed under a range of conditions were analysed by differential centrifugation using a CPS Disc Centrifuge UHR for particle sizing (CPS Instruments, Inc., USA). For this, Se⁰ particles were resuspended by sonication for 3 minutes in 0.1% aqueous sodium dodecylsulfate (SDS). Samples were then injected into the disc centrifuge running at 20,000 rpm with a sucrose gradient composed of a series of sucrose solutions between 24% and 8% concentration. A 0.377 µm PVC standard was measured to ensure accuracy of the results. CPSV95 software was used for data collection and analysis. This technique is ideal for particle size characterisation of relatively large (~100nm) spherical particles of a known composition.

Investigations into the long-term stability of amorphous Se⁰ precipitates was carried out by X-ray diffraction on air-dried, glass slide mounted samples with a Bruker D8 Advance with a Cu Kα radiation source (1.542Å).

Amorphous red Se⁰ was also prepared abiotically by the reduction of 1mM sodium selenite by 4mM reduced glutathione, which was then titrated with 1N NaOH until a red precipitate formed, as described by Johnson et al (22).

3. **Results**

3.1. **Growing cell experiments**

Cells of *G. sulfurreducens* were unable to conserve energy for growth by coupling the oxidation of H₂ or acetate to the reduction of Se⁴⁺ or Te⁴⁺. However, the small number of cells present in the initial inoculum formed red and black precipitates in the Se and Te amended bottles, respectively, consistent with published reports of reduction to elemental forms of the metalloids (3, 40, 41). Accordingly, experiments to determine maximum inhibitory concentration (MIC) values of Se⁴⁺ and Te⁴⁺ for *G. sulfurreducens* were conducted, and direct cell enumeration showed that the organism was unable to grow using fumarate
as an electron acceptor at concentrations >10 μM of either the Se or Te oxyanions. IC analysis of the Se or Te amended solutions following incubation saw no significant decrease in soluble Se\textsuperscript{IV} or Te\textsuperscript{IV} at any of the concentrations tested, suggesting that the concentrations of Se\textsuperscript{IV} or Te\textsuperscript{IV} reduced to form the observed Se and Te precipitates was below detections limits. The black Te-containing precipitates were characterised using TEM (figure 1). EDX analysis confirmed that the precipitates were elemental Te as no significant concentrations of possible counterions were detected. Two discrete morphologies were discernable (figure 1B); acicular Te nanorods ranging in size from 50-200nm in length and 10-20nm in diameter and Te\textsuperscript{0} nanospheres less than 10nm in diameter arranged into large spherical clusters and found both externally to the cell and localised at the cell surface.

SEM was used to characterise the red Se-containing precipitates showing amorphous, smooth spheres typically 100-200nm in diameter (figure 1C and D). The red precipitates were an elemental Se phase in accordance with previous findings as determined by EDX (19, 41). Se\textsuperscript{0} nanospheres were found both extracellularly and attached to external surfaces of cells.

Despite the calculated low MIC values (~10μM Se\textsuperscript{IV} or Te\textsuperscript{IV}) resting cell cultures of \textit{G. sulfurreducens} have been shown to be capable of the reduction of up to 6.5mM Se\textsuperscript{IV}, and a 15KDa c-type cytochrome was identified by LC-MS/MS as being associated strongly with the Se\textsuperscript{0} precipitates (41). \textit{S. oneidensis}, also known to contain significant numbers of c-type cytochromes, is capable of growth and reduction in the presence of >2mM Se\textsuperscript{IV} (24, 41). Direct comparisons by Pearce \textit{et al} (41) between \textit{G. sulfurreducens} and \textit{S. oneidensis} showed that the former was able to reduce Se\textsuperscript{IV} at a higher rate, which was attributed to higher cytochrome content. The growing cell experiments described here show that \textit{G. sulfurreducens} is incapable of growth in >10μM concentrations of Se\textsuperscript{IV} or Te\textsuperscript{IV}, which is likely attributable to the toxicity of these oxyanions rather than an inability to reduce them. A review of bacterial Se and Te detoxification mechanisms by Zannoni \textit{et al} (51) list four possible detoxification pathways, and a painter-type reaction involving reduced thiol compounds is described for both
oxyanions. The reduction of Se$^{IV}$ by reduced cellular thiols, primarily reduced glutathione, leads to the formation of a selenodiglutathione complex along with reactive superoxide species. Selenodiglutathione can then be acted upon by either glutathione reductase or thioredoxin reductase, ultimately leading to the regeneration of the initial reduced glutathione and an elemental Se precipitate.
An analogous pathway is suggested for Te\(^0\) precipitate formation (51). A comparison of the genomes of *S. oneidensis* MR-1 and *G. sulfurreducens* PCA reveals that both glutathione reductase and thioredoxin reductase are present in *S. oneidensis* MR-1; whereas only thioredoxin reductase is present in *G. sulfurreducens* PCA. Thus, the inability of *G. sulfurreducens* to grow in >10\(\mu\)M concentrations of Se\(^{IV}\) whilst *S. oneidensis* can grow in Se\(^{IV}\) concentrations exceeding 2mM (24) could indicate the importance of glutathione reductase in Se and Te oxyanion detoxification mechanisms.

Baesman *et al* (3) and Oremland *et al* (40) report on the formation of both internal and external Te\(^0\) and Se\(^0\) precipitates by *Bacillus selenitireducens*, *Sulfurospirillum barnesii* and *Selenihalanaerobacter shriftii*. They hypothesise that dissimilatory Te\(^{IV}\) and Se\(^{IV}\) reduction results in the formation of extensive external precipitates, and that the more limited internal precipitates form as a result of a detoxification mechanism. The findings of Wang *et al* (48) are in agreement with these results, with both intra- and extracellular Se\(^0\) and Te\(^0\) deposits produced by *Escherichia coli*. It has also been shown that c-type cytochromes are involved in bacterial reduction of Te\(^{IV}\) (45) and Se\(^{IV}\) oxyanions (1, 41), and therefore it is possible that the two morphologies of Te\(^0\) noted in this work are linked to dissimilatory reduction involving outer membrane cytochromes and internal detoxification mechanisms. Only a single morphology of Se\(^0\) was noted.

### 3.2. Resting cell experiments

Resting cell cultures of *G. sulfurreducens* developed a deep black precipitate when incubated with sodium tellurite (1mM). TEM images of these precipitates are shown in figure 2, and indicate an average particle width of 10-15nm and up to 70nm in length, similar to those described for cultures of *G. sulfurreducens* incubated in growth medium. The particles are also shown to display a high degree of crystallinity (figure 2B), and EDX analysis indicates no likely counterion, suggesting a pure Te\(^0\) phase. No other Te nanoparticle morphology was observed, unlike in the growing cell experiments, suggesting that, under resting cell conditions, only one reduction mechanism occurs. The
most likely mechanism involves dissimilatory reduction by c-type cytochromes, as they have been detected in association with products of reduction by *G. sulfurreducens* cells under similar experimental conditions (12, 41).

*Figure 2.* TEM of Te⁶ nanorods produced by resting cell cultures of *G. sulfurreducens* showing (A) the typical particle size and (B) the well developed crystalline nature of the nanorods. The X-ray emission spectra shows the presence of high concentrations of Te, with Cu reading originating from the Cu grid used and the S from the 20mM MOPS buffer solution.
The reduction of Se\textsuperscript{IV} by pre-grown ‘resting cell’ suspensions of \textit{G. sulfurreducens} alleviates the problem of acute toxicity of the oxyanion and results in the production of significant quantities of Se\textsuperscript{0} particles. Due to the technological, chemical and industrial applications of selenium, the ability to tune Se\textsuperscript{0} nanoparticles to a predetermined desired size and morphology by varying the initial incubations conditions warrants investigation. It has been shown that particle size and surface area control the effectiveness of the Se\textsuperscript{0} particles for specific applications, such as the capture and retention of toxic Hg\textsuperscript{0} vapour (19, 22).

\textbf{Figure 3.} Reduction of 1 mM Se\textsuperscript{IV} (as percentage of total) (A) pH5.5, stationary phase cells, H\textsubscript{2} electron donor, (B) pH8.5, stationary phase cells, H\textsubscript{2} electron donor, (C) pH7 log phase cells, acetate electron donor, (D) pH7, log phase cells, H\textsubscript{2} electron donor, (E) pH7, stationary phase cells, H\textsubscript{2} electron donor, (F) pH7, stationary phase cells, acetate electron donor and (G) pH7, stationary phase cells, acetate electron donor incubated under microaerophilic conditions. Light grey bars are incubated with 2\textmu M AQDS, dark grey bars without.
To investigate the impact of a range of incubation parameters on the formation of the Se⁰ precipitates, a matrix of experiments was designed using an inoculum concentration of 3.6 x10⁸ cells ml⁻¹. The total amount and rate of Se⁴ reduced after 96 hours incubation was measured by IC and is shown in figures 3 and 4, respectively, with the particle size distributions shown in figure 5.

Figure 4. Rates of Se⁴ reduction under a range of process conditions, with and without AQDS.

Incubation of stationary phase G. sulfurreducens cells with H₂ at pH5.5 led to 14% removal of Se⁴, compared to 66% reduction at pH8.5 and 87% reduction at pH7. Se⁴ was reduced at a rate of 17.5μM hr⁻¹ at pH7, and 20.4μM hr⁻¹ at pH8.5, with average particle sizes of 169nm and 193nm, respectively. Both sodium acetate and H₂ were tested to see if either could be coupled to Se⁴ reduction by G. sulfurreducens at pH7, with 68% and 87% reduction, respectively. Se⁴ was reduced at 17.5μM hr⁻¹ with H₂ and 16.1μM hr⁻¹ with acetate, and average particle diameters were 169nm with H₂ and 162nm when acetate was used. Comparisons between G. sulfurreducens cells isolated during exponential
growth (log phase) or immediately following exponential growth (stationary phase) in defined medium amended with acetate and fumarate showed that stationary phase cells were able to reduce 87% of the Se\textsuperscript{IV}, whereas log phase cells were only able to reduce 50%. Comparisons of the rate of Se\textsuperscript{IV} reduction shows that stationary phase cells reduce Se\textsuperscript{IV} more rapidly (17.5µM hr\textsuperscript{-1}) than cells isolated at log phase (13.6µM hr\textsuperscript{-1}), but form larger particles (average particle diameters of 162nm and 144nm, respectively).

Figure 5. Particle sizing of Se\textsuperscript{0} precipitates, without (left) and with (right) AQDS as a function of Se\textsuperscript{IV} reduction rate.

The electron shuttling compound AQDS has been shown to increase markedly the rate and total amount of Se\textsuperscript{IV} reduction (41), and resting cell experiments during this work were duplicated with the addition of 2µM AQDS. AQDS amended cultures reduced a higher percentage of the total Se\textsuperscript{IV} under all conditions tested with stationary phase cells at pH7 using either H\textsubscript{2} or acetate as the electron donor reducing 100% of amended Se\textsuperscript{IV}. The maximum rate of reduction of Se\textsuperscript{IV} was observed by G. sulfurreducens incubated with H\textsubscript{2} at pH8.5 at 24µM hr\textsuperscript{-1}. Incubations in the presence of AQDS also saw greater average
particle sizes and wider particle size distributions under all conditions than in experiments without the electron shuttle. The effect of low concentrations of O₂ were also determined for stationary phase cells at pH7 using acetate as the electron source and made no significant difference in experiments without AQDS, however incubations with AQDS saw a 20% decrease in total Se⁴⁺ reduced compared to strictly anaerobic incubations.

To characterise the affinity of the whole cells to Se⁴⁺, the Michaelis Constant (K_M) for the reaction was determined empirically; the K_M was calculated to be 1.06mM with a V_MAX of 158μM hr⁻¹.

Particle characterisation of the precipitates formed by G. sulfurreducens under all conditions by SEM and TEM showed clusters of smooth, amorphous, spherical Se precipitates in the range 100-200nm in diameter (figure 6), confirmed as elemental Se by EDX analysis. Se⁰ nanospheres were found in close association (figure 6A) and boundaries between Se⁰ nanospheres were often not visible with particles merging into one another (figure 6B). XRD analysis of the precipitates formed by G. sulfurreducens at pH7 showed that nanospheres remained stable both in suspension and also when dried for over a year following formation, in contrast to abiotically synthesised red Se⁰ which crystallised when dried to the black trigonal form within 53 days (figure 7). The average and peak frequency of particle size distributions of the Se⁰ particles determined by disc centrifugation (figure 5) shows a strong positive correlation with the rate of Se⁴⁺ reduction in the absence of AQDS (r² of 0.74 and 0.96, respectively), whilst those incubated with AQDS show no correlation (r² of 0.02 and 0.20, respectively).

In contrast to the pH7 experiments, incubations at pH8.5 saw a gradual alteration in the colour of the original red Se⁰ precipitates to black during the experiment, and TEM analysis revealed this was due to the formation of bundles of crystalline Se⁰ nanorods (figure 6D, E).
Figure 6. Se$^0$ nanoparticles produced by resting cell G. sulfurreducens cultures incubated with 1mM Se$^IV$; (A) SEM of particles produced by log phase cells at pH7 with AQDS and H$_2$, (B) the close relationship between Se$^0$ particles, (C) indication of a
coating layer over \( \text{Se}^0 \) precipitates formed in the absence of AQDS; inset, increased magnification, (D) nucleation of crystalline \( \text{Se}^0 \) at pH8.5 within a cluster of amorphous nanospheres, with the residual amorphous \( \text{Se}^0 \) outline visible, (E) direct attachment of amorphous \( \text{Se}^0 \) to the crystalline phase, (F) SAED of the crystalline \( \text{Se}^0 \) showing it to be the trigonal polymorph.

4. Discussion

The ability of resting cell cultures of \( G. \text{sulfurreducens} \) to produce large quantities of \( \text{Se}^0 \) precipitates at concentrations two orders of magnitude higher than calculated MIC values is notable. Clearly the enzymatic machinery needed within the cells for these reductive transformations are not impacted severely by the toxicity of the metalloids, even though much lower concentrations can completely inhibit growth of the organism. Process parameters such as temperature and pH do, however, impact on \( \text{Se}^{IV} \) reduction by \( G. \text{sulfurreducens} \). The total amount of \( \text{Se}^{IV} \) reduction (without shuttling compounds) was highest for the pH7 incubations, consistent with optimal culture conditions for this neutraphilic organism. As reported previously, \( G. \text{sulfurreducens} \) was able to utilise both acetate and \( \text{H}_2 \) as an electron donor (8, 9, 41, 50), coupling this to reduction of \( \text{Se}^{IV} \). Differences in the rate of \( \text{Se}^{IV} \) reduction are likely attributable to the differences in reaction kinetics between \( \text{H}_2 \) and acetate utilisation (8), and are in accordance with previous findings (41). Recent research published by Lee et al (28) and Tam et al (44) established that variations in \( \text{O}_2 \) and biomass concentrations could lead to decreased particle size and size distributions, respectively, for \( \text{Se}^0 \) precipitates produced by \( \text{Shewanella} \) sp HN-41. Analysis of the size distribution of particles produced by \( G. \text{sulfurreducens} \) shows that these nanoparticles can also be manipulated by varying initial conditions.

Nanospheres of red amorphous elemental selenium (figure 6A) are produced by \( G. \text{sulfurreducens} \), and are similar in appearance to bacterial \( \text{Se}^0 \) formed under
both anaerobic and aerobic conditions (21, 23, 24, 28, 40, 41, 43, 44, 48). The close proximity of neighbouring Se$^0$ nanospheres (figure 6B) is interesting, as the original spherical morphology remains but without a clearly defined boundary between the particles. It is unclear from these findings whether coalescence occurs as a result of electrostatic attraction or owing to the close proximity of fixed nucleation points.

![X-ray diffraction patterns](image)

*Figure 7. X-ray diffraction patterns showing the initial biologically precipitated Se$^0$ (B$_0$) remaining as an amorphous phase after 58 days in aerobic aqueous suspension (B$_{58}$), whilst abiotically synthesised Se$^0$ had crystallised to the trigonal form by 53 days after formation (A$_{53}$).*

XRD analyses (figure 7) show that biologically synthesised amorphous Se$^0$ is more stable, either as a dried Se$^0$ powder or in aerobic aqueous suspension, comparative to abiotically prepared amorphous Se$^0$. These findings are concordant with those of investigations into Se$^0$ precipitates by a range of bacteria (19, 23, 40, 41). Research into the chemical synthesis of amorphous Se$^0$ in the presence of proteins by Kessi et al (23), Valueva et al (46), Johnson et al (22) and Dobias et al (17) all show that stable, smooth, spherical, amorphous red Se$^0$ nanoparticles are synthesised only with the addition of proteins. Valueva et al (46) and Johnson et al (22) hypothesise that the protein bovine
serum albumin (BSA) forms a stabilising multilayer coating on the surface of a Se⁰ nuclei. A coating layer is visible on the bacterial Se⁰ precipitates formed by *G. sulfurreducens* in this study (figure 6C), and may act to stabilise the Se⁰ particles as noted previously, supporting the findings of strongly associated cytochromes by Pearce *et al* (41).

The electron shuttling compound AQDS increased the total amount and rate of Se⁴ reduction in all anaerobic applications (figures 3 and 4), in accordance with the results of Pearce *et al* (41) for *G. sulfurreducens* and Wang *et al* (48) for *E. coli*. The relative decrease in Se⁴ reduction in the presence of AQDS under microaerophilic conditions compared to anaerobic incubations indicates that O₂ may serve as an alternative electron acceptor for AQDS, thus decreasing its effectiveness.

Two distinct particle size ranges can be produced in the presence or absence of the electron shuttle AQDS (figure 5). In conjunction with the findings of Pearce *et al* (41), who suggested that outer membrane bound c-type cytochromes are involved in Se⁴ reduction, and the findings of Wang *et al* (48), who found that an increased fraction of external Se⁰ precipitates were seen when a range of electron shuttles were added to *E. coli* cultures amended with Se⁴, it can be inferred that the difference in the size ranges is a result of different mechanisms of formation; (i) Se⁰ precipitates formed as a result of direct contact with the outer cell membrane bound c-type cytochromes and (ii) Se⁰ precipitates formed via reduction by AQDS. A narrower size distribution is found in Se⁰ precipitates formed directly associated with *G. sulfurreducens*, indicating that the cell surface may be limiting particle growth. The difference in particle size distribution in the absence of AQDS is related to differences in the rate of Se⁴ reduction; particles formed at a higher rate display a wider particle size range and larger average particle diameter (figure 5).

TEM and SEM analysis of the Se⁰ precipitates formed at pH8.5 show the formation of a crystalline ‘nanowire’ phase at ambient temperatures, discernable in the particle size distribution results as a skew towards larger particle sizes and especially apparent for those Se⁰ particles formed in the presence of AQDS (figure 5). The transformation to the crystalline trigonal form
is in agreement with Ho et al (21) and Cheng et al (11), who found that dissolution of amorphous Se\(^0\) at alkaline pH values led to the formation of Se\(^0\) crystals with growth preferentially elongated along the [001] direction. In this study, the crystalline phase nucleated from the amorphous Se nanospheres (figure 6D). The close proximity of amorphous Se\(^0\) nanospheres that are tapering into crystalline Se\(^0\) (figure 6E) suggest that Se dissolution and re-precipitation occurs over a short distance. The observation that Se\(^0\) crystallisation occurs to a greater extent in the presence of AQDS supports the hypothesis that extracellular, abiotic reduction of Se\(^{IV}\) to Se\(^0\) by AQDS occurs, and subsequently leads to Se\(^0\) nanoparticles which display a lower stability compared to particles formed in direct contact with cells.

5. Conclusion

There is currently intense interest in developing novel techniques for the synthesis of a range of nanoscale semiconductor and photovoltaic materials that may have uses in the industrial, electronic and chemical sectors. Bacteria can synthesise nanoscale materials at ambient temperatures and pressures representing a cost efficient, highly scalable technique that can be undertaken without the use of hazardous intermediary chemicals. The use of bacteria to selectively produce these nanomaterials also provides a link to the bioremediation of metalloid contaminated waste streams (30, 31).

This work shows that Geobacter sulfurreducens cannot utilise Se\(^{IV}\) and Te\(^{IV}\) for growth, but the small number of cells in the initial inoculum are able to reduce a very limited quantity of these oxyanions, resulting in the production of Se\(^0\) nanospheres 100-200nm in diameter and a combination of Te\(^0\) nanorods 50-200nm in length with clusters of 20nm nanospheres, respectively. Resting cell cultures of Geobacter sulfurreducens can produce large quantities of crystalline Te\(^0\) and amorphous Se\(^0\) at concentrations two orders of magnitude above the MIC value (10 \(\mu\)M). Se\(^0\) precipitates as 100-200nm diameter amorphous nanospheres in close proximity and spatially separated from the cells. Biological Te\(^0\) precipitates are crystalline nanorods up to 70nm in length. The bacterially
derived amorphous Se\textsuperscript{0} nanospheres displayed increased stability when compared with abiotically synthesised Se\textsuperscript{0}.

This research shows that by altering the incubation conditions of \textit{G. sulfurreducens}, it is possible to tailor the resulting Se\textsuperscript{0} biological precipitates to predetermined characteristics with respect to particle size distribution and morphology. Se\textsuperscript{0} particles formed in direct contact with the \textit{G. sulfurreducens} cell surface, which contains an abundance of groups capable of coordinating metals and metalloids, as well as outer membrane bound c-type cytochromes implicated in electron transfer to Se and Te oxyanions, exhibit a narrow size distribution and enhanced stability.

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Chapter 6

Research paper 3

Use of biogenic and abiotic elemental selenium nanospheres to sequester elemental mercury released from mercury contaminated museum specimens


Use of biogenic and abiotic elemental selenium nanospheres to sequester elemental mercury released from mercury contaminated museum specimens

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A B S T R A C T

Mercuric chloride solutions have historically been used as pesticides to prevent bacterial, fungal and insect degradation of herbarium specimens. The University of Manchester museum herbarium contains over a million specimens from numerous collections, many preserved using HgCl₂ and its transformation to Hg⁰ represents a health risk to herbarium staff. Elevated mercury concentrations in work areas (~1.7 μg m⁻³) are below advised safe levels (<25 μg m⁻³) but up to 90 μg m⁻³ mercury vapour was measured in specimen boxes, representing a risk when accessing the samples. Mercury vapour release correlated strongly with temperature. Mercury salts were observed on botanical specimens at concentrations up to 2.85 wt% (bulk); XPS, SEM–EDS and XANES suggest the presence of residual HgCl₂ as well as cubic HgS and HgO. Bacterially derived, amorphous nanospheres of elemental selenium effectively sequestered the mercury vapour in the specimen boxes (up to 19 wt%), and analysis demonstrated that the Hg⁰ was oxidised by the selenium to form stable HgSe on the surface of the nanospheres. Biogenic Se⁰ can be used to reduce HgCl₂ in long term, slow release environments.

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1. Introduction

The preservation of botanical specimens in museums across the world has traditionally proven difficult. A wide range of organic and toxic metal biocides have been used to deter the onset of decay caused by bacteria, fungi, insects and rodents; the efficiency of these treatments can be seen by the excellent state of preservation in many specimens today. The use of mercury salt solutions in the preservation of botanical specimens goes back to 1687, and continued in the UK up until the 1980s [1,2], ultimately giving way to organic pesticides including naphthalene, p-dichlorobenzene, thymol, lindane and DDT [2,3]. The preparation of mercury bearing pesticide solutions is detailed by Briggs et al. [4] and a concentration of 30 g l⁻¹ HgCl₂ and 30 g l⁻¹ phenol dissolved into methylated spirit was used in the Cambridge University Herbarium, UK. The low rate of sublimation of HgCl₂ and careful storage ensure that botanical specimens dating back hundreds of years still hold significant concentrations of Hg; as Hg⁰, residual HgCl₂, HgS and 2HgO·HgS [2,4–10]. Amalgamation of botanical collections into large herbaria, each having undocumented preservation techniques, has lead to inhomogeneous distribution of mercury contaminated specimens within collections. Staining of sample mounting paper due to the formation of crystalline Hg phases has been used to identify the presence of Hg-bearing compounds by fluorescence using UV irradiation [2].

Health risks associated with Hg-bearing botanical samples are not confined to dermal contact with contaminated specimens; Briggs et al. [4] report the evolution of Hg⁰ vapour leading to elevated local Hg concentrations of 25 μg m⁻³ at the Cambridge University Herbarium. Investigations in other herbaria indicate that this problem is widespread [8,9]. The concentration of Hg⁰ is dependent on ambient temperature; Oyarzun et al. [8] show that the Hg vapour concentration in the MAF Herbarium, Spain rises from 404–727 ng m⁻³ in late winter (23 °C) to 748–7797 ng m⁻³ in early summer (31 °C). Oyarzun et al. [8] also show that in a well insulated herbarium such as the relatively modern MA Herbarium, Spain, Hg⁰ concentrations exceeded 40 μg m⁻³.

Regulatory standards for workplace exposure limits have been summarised by Baughman [11], with guideline exposure limits varying between 25 μg m⁻³ and 100 μg m⁻³ Hg. It is also noted that children are much more susceptible to Hg toxicity than adults, which is of concern for public access museums.

The transformation mechanism of the relatively stable HgCl₂ into Hg⁰ is not well understood. Oyarzun et al. [8] surmised that microbial enzymatic reduction of Hg⁰ to Hg⁰ as part of a Hg detoxification mechanism may be responsible, and work by Roane and...
Snelling [12] has identified bacteria isolated from museum specimens that display a high Hg\(^{2+}\) tolerance (in excess of 10 mg l\(^{-1}\)), which may also be used as a possible remediation strategy. Complicating this process, however, is the presence of a range of other pesticides often found alongside Hg.

Briggs et al. [4] and Oyarzun et al. [8] both report that an increase in ventilation significantly drops the Hg\(^0\) content of the air. There are circumstances where increasing ventilation cannot be considered due to climate, expense or positioning of the herbarium within buildings, and so an alternative method for decreasing the Hg content of herbarium and museum specimens must be identified.

Johnson et al. [13] studied the capture of Hg\(^0\) by nanoscale sorbents, and note that elemental α-Se nanoparticles sequester Hg more efficiently than many commercially available sorbents. Se nanoparticles can be produced biogenically by bacterial reduction of soluble Se oxyanions. Se pollution, in the form of the oxyanions selenate [SeO\(_4^{2-}\)] and selenite [SeO\(_3^{2-}\)], is associated with waste materials from a broad spectrum of anthropogenic operations, including mining, agricultural, petrochemical, and industrial manufacturing operations [14]. Water-soluble forms of selenium can be microbiologically reduced to elemental selenium nanoparticles, which are less bioavailable and generally less toxic than other selenium species. The elemental selenium nanoparticles can potentially be separated from the aqueous waste stream [15]. The biologically recovered selenium nanoparticles can then be used in subsequent applications, such as the sequestration of elemental mercury released from mercury contaminated museum specimens, to offset the cost of the biological treatment [16]. This highlights how biomineralisation approaches can be applied to convert metal-containing wastes into new nanomaterials for environmental protection [17].

This research reveals the nature and extent of Hg contamination associated with museum specimens and measurement of Hg content of airspaces at the herbarium of the Manchester museum (The University of Manchester, UK), which has a botanical collection approaching a million specimens from all over the world, representing collections spanning hundreds of years. The collection is subdivided into British and European collections, as well as several smaller collections. The level of Hg contamination is inhomogeneous and poorly documented. This work also investigates the use of Se bionanominerals, which display an increased stability in comparison with their chemically synthesised counterparts [14,18,19] for the capture of Hg\(^0\) released from specimens within herbaria.

2. Materials and methods

All chemicals used were of analytical grade and obtained from Sigma–Aldrich (UK).

2.1. Determination of herbarium air Hg\(^0\) concentration

The Hg content of the herbarium air was determined using a portable mercury vapour indicator (MVI, Shawcity, Faringdon, UK). The Hg\(^0\) concentration of the air was determined around the central workspaces of the herbarium and in sample boxes from a number of collections. The boxes analysed were chosen at random and were analysed immediately for Hg\(^0\) concentration.

2.2. Sampling

The museum’s herbarium specimens are mounted onto A3 sized cardboard sheets and stored in fabric lined cardboard boxes on shelves within the herbarium, as shown in Fig. 1.

Samples from the British and European collections were analysed for Hg concentration and oxidation state. Due to the irreplaceable nature of the specimens, milligram sized samples were taken and non-destructive analysis techniques (X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM) coupled to energy dispersive spectroscopy (EDS) and X-ray absorption spectroscopy (XAS)) were used prior to chemical dissolution to determine bulk Hg values. Samples include leaf segments, mounting paper and plant sample debris (Table 1).

2.3. Mercury sequestration experiments

The Hg absorption capacity of dry powders of both biogenic and abiotic Se\(^0\) was tested. Biogenic red elemental Se\(^0\) was formed by the reduction of Na\(_2\)SeO\(_3\) by Geobacter sulfurreducens coupled to the oxidation of H\(_2\). G. sulfurreducens (ATCC 51573) was obtained from the Geomicrobiology Laboratory (University of Manchester) collection and grown to late log phase in modified freshwater medium amended with sodium acetate and fumarate as the electron donor and acceptor, respectively [20]. Cells

![Fig. 1. Stacks of herbarium samples in containing boxes (left) and a typical herbarium specimen mounted on A3 paper with dark staining visible towards the top (right).](image-url)
were harvested, washed and re-suspended in 20 mmol l\(^{-1}\) 3-(N-morpholino)propanesulfonic acid (MOPS) buffer at pH 7. Aliquots of the cell suspension were used to inoculate sterile, anaerobic solutions (final OD\(_{600}\) ~0.2) of 1 mmol l\(^{-1}\) Na\(_2\)SeO\(_3\) in 20 mmol l\(^{-1}\) MOPS amended with 2 μmol l\(^{-1}\) of the electron shuttling compound antheraquinone-2,6-disulphonate (AQDS). The headspaces of the bottles were replaced with H\(_2\) as an electron donor. Cultures were incubated at 30°C and red Se\(^0\) was precipitated. To produce abiotic Se\(^0\), a solution containing 1 mmol l\(^{-1}\) Na\(_2\)SeO\(_3\) and 4 mmol l\(^{-1}\) glutathione (reduced) was titrated against a 1N NaOH solution until the formation of red elemental Se was observed [13]. For the sequestration experiments, biogenic and abiotic red Se\(^0\) suspensions (5 ml) were filtered through 0.22 μm pore filters, collected from lining of box and allowed to air dry.

### 2.3.2. Sealed environment experiments

The ability of the Se\(^0\)-containing membrane filters to sequester Hg\(_0\) was assessed under idealised laboratory conditions. Se\(^0\)-laden membrane filters were inserted into air tight 11 jars for 1 week at constant temperature (19°C). HgCl\(_2\)/SnCl\(_2\) solutions served as the Hg\(^2+\) source for these experiments. HgCl\(_2\) concentrations ranged from 10 ppb to 10 ppm and SnCl\(_2\) concentrations were kept in the ratio 1:100 Hg:Sn. After 1 week, Se\(^0\) membrane filters were removed from the sealed jars and stored at −80°C prior to acid digestion and Hg determination using ICP-MS.

### 2.3.3. In situ experiments

Biogenic Se\(^0\)-containing membrane filters were inserted into herbarium specimen boxes known to contain significant Hg\(_0\) concentrations (>25 μg m\(^{-3}\)). The samples were left for four weeks, during which the time ambient temperature rose from 19°C to 24°C due to weather conditions. Following collection, samples were stored at −80°C until acid digestion for Hg determination.

### 2.4. Hg-species characterisation

Samples described in Table 1 and Se\(^0\)-containing membrane filters from the mercury sequestration experiments were analysed by SEM using a Philips XL30 FEG-ESEM with elemental analysis by EDAX Gemini EDS. All samples were mounted onto adhesive coated-Al stubs to remove interference in Se determination caused by overlapping Al emission peaks. XRD of mounting paper samples (Table 1) was carried out on a Bruker D8 Advance with a Cu-Kα source. Spectra were compared to samples published by the International Centre for Diffraction Data (ICDD).

XAS was performed on samples described in Table 1 and Se\(^0\)-containing membrane filters at the Diamond Light Source, Oxfordshire, UK, on the core XAS beamline B18 where samples were analysed at the Hg L\(_3\) absorption edge at 12.287 keV. HgS standards were used to monitor the energy of the spectra.

Acid digestion using \textit{aqua regia} (1 ml) with a molar ratio 1:4 HCl:HNO\(_3\) was used to determine the concentration of Hg in all sample materials. Excess HNO\(_3\) was used to encourage formation of Hg\(^{2+}\) cations to decrease losses due to volatility. Samples were run on an Agilent 7500cx ICP-MS, with quoted Hg detection down to 10 ppt. A 10 ppm Au solution was run concurrently to inhibit Hg retention. To ensure complete recovery of Hg, a 1 ml HF–HCl–HNO\(_3\) digestion (5:1:4) was used on any remaining material. The HF was neutralised using a 4 wt% solution of H\(_2\)BO\(_3\). Owing to the necessary high dissolved solid content, these samples were analysed using a Perkin-Elmer Optima 5300 dual view ICP-AES.

### 3. Results and discussion

#### 3.1. Determination of herbarium air Hg\(_0\) concentration

The Hg\(_0\) concentration of air around the work area was 1.7 μg m\(^{-3}\) at 21°C, which did not significantly alter throughout the herbarium. These results are similar to those observed in previous studies by Oyarzun et al. [8] and Kataeva et al. [9], which
recorded readings of up to 0.7 μg m⁻³ and 1.1 μg m⁻³ under similar conditions (21–23 °C).

The Hg⁰ concentration within the specimen boxes was determined for 70 boxes from 5 collections, of which 55 came from either the British or European collections. The Hg⁰ concentrations were highly variable with the highest concentrations in the European collection with Hg⁰ ranging from below detection limits up to 52 μg m⁻³, with 5 boxes tested recording >25 μg m⁻³ (Fig. 2). The British collection was largely free of significant Hg-contamination; however the Hg⁰ readings for the Rubus sp. type samples box were in excess of 40 μg m⁻³ (data not shown).

High Hg⁰ concentrations were not observed outside of the specimen boxes prior to opening, indicating that the boxes inhibit the release of Hg into the working environment.

3.2. Characterisation of museum Hg-contaminated specimens

3.2.1. Electron microscopy and X-ray diffraction

Fig. 3A shows representative SEM images of samples collected from specimen boxes containing grains of sample debris (sample 4). EDS revealed that most of the samples examined did not have significant quantities of Hg. However, analysis of one particular grain (Fig. 3B and C), showed ~1 μm particles composed of ~87 wt% Hg with the counter ions S²⁻ and Cl⁻. The S component could not be quantified due to the overlap of the Hg M and S K emission lines, but the Hg wt% observed is similar to that expected from pure HgS (86 wt% as opposed to 74 wt% and 93 wt% Hg in HgCl₂ and HgO, respectively).

Samples of the A3 specimen mounting card (samples 2 and 8) were analysed by XRD (Fig. 4), indicating the presence of crystalline Hg phases. Sample 2 displayed peaks corresponding to unaltered HgCl₂ (ICDD PDF No. 00-026-0315) whilst sample 8 displayed a reflection at 26.4° 2θ, suggesting crystalline metacinnabar (ICDD PDF No. 00-006-0261). Hawks et al. [6] and Purewal et al. [2] have previously observed crystalline HgO, HgS and HgCl₂ phases on the mounting card.

3.2.2. XPS and XAS analysis

XPS analysis was used to identify the chemical forms of Hg present on the sample surface. Fig. 5 shows XPS spectra obtained from a Eucalyptus sp. leaf (sample 7) historically treated with Hg-based pesticides. XPS analysis showed that Hg was inhomogeneously distributed, with some areas falling below detection limits and others up to 1100 ppm. For the high Hg-containing areas, the

Fig. 2. Hg⁰ concentrations within herbarium specimen boxes.
Fig. 4. XRD data from analysis of samples 2 and 8 (mounting card). (A) Indicating remnant HgCl₂ and (B) diffraction peaks suggesting metacinnabar.

Fig. 5. XPS wide scan and inset, narrow scan spectra of *Eucalyptus* sp. leaf. Peaks correlating to Hg, C, N and O are all clearly discernable. Hg concentration determined to be ∼1100 ppm.

binding energy for the Hg 4f emission was 101 eV. Comparison with published standards indicates that the Hg could be present as HgS, HgCl₂, HgO or as an organo-mercury compound, but precludes the presence of Hg⁰ [24].

Fig. 6. XANES Hg L₃ absorption edge spectra obtained from (A) *Eucalyptus* sp. leaf; (B) box dust (sample 4) and (C) Se-laden polycarbonate membrane filter exposed to Hg⁰. Inset: 1st derivatives of the XANES spectra.
The XANES spectrum derived from the *Eucalyptus* sp. leaf is presented in Fig. 6A, with the 1st derivative of the XANES spectrum inset. An IPD value of 7.88 eV was recorded for the first derivation, consistent with published HgS species [25]. Linear combination fitting of published HgS standards [23,25,26] suggests the XANES profile is consistent with metacinnabar, concordant with XRD findings. An IPD value of 7.89 eV was derived from the XANES analysis (Fig. 6B, inset) of the Hg-rich dust (sample 4), and is concordant with the EDS indicating the presence of a dominant HgS phase. The presence of multiple peaks in the 1st derivation of all XANES spectra obtained shows no Hg\(^0\) is present in these samples.

3.2.3. Acid digestion of specimens in Table 1

Total extraction of Hg from the museum specimens was used to determine absolute Hg concentrations. The results of the sequential acid extractions using *aqua regia* and HF-*aqua regia* showed that Hg concentration in the specimens varied greatly (Table 1). Interestingly, the digests show that the *Eucalyptus* sp. leaf and plant stem (samples 7 and 9) do not contain significant concentrations of Hg (0.08 and 0.07 wt%). Dust and debris collected from two of the specimen wrapping sheets (samples 1 and 3) show Hg concentrations of ~0.2 wt%, but debris collected from the box containing the specimens (sample 4) had a Hg concentration of 2.85 wt%, over an order of magnitude larger. These results highlight the heterogeneity of Hg contamination within the specimens and may relate to varying pesticide coating practices. Hg contamination was found in all samples.

3.3. Mercury sequestration experiments

3.3.1. Selenium nanoparticle and filter characterisation

Selenium impregnated membrane filters were characterised prior to use in laboratory experiments by ICP-AES/-MS, SEM with EDS and particle sizing. Total acid digests showed that membrane filters were loaded with 500–600 \(\mu\)g of Se per filter for both abiotic and biogenic Se nanoparticles. SEM analyses of the loaded membranes prior to experimentation show smooth, spherical particles approximately 100–200 nm in diameter (Fig. 7A). EDS analyses of the Se phases (Fig. 7Di) show a dominant Se L edge peak at 1.4 eV along with smaller C and O emissions from the filter material, indicating a pure Se phase. Size distribution information was obtained (Fig. 8E) and was concordant with SEM analyses show particles ranging from 40 to 700 nm, similar to that as reported by Johnson et al. [13]. Size distribution information was used to calculate surface area assuming spherical particles; abiotic Se\(^0\) had a surface area of 11.64 m\(^2\) g\(^{-1}\) and biogenic Se\(^0\) 9.64 m\(^2\) g\(^{-1}\) equating to 5.8–7.0 \(\times\) 10\(^{-3}\) m\(^2\) and 4.8–5.8 \(\times\) 10\(^{-3}\) m\(^2\) per filter, respectively.

3.3.2. Open system experiments

Biogenic Se\(^0\)-containing membrane filters were assessed using the modified CVAAS to follow the evolution of Hg\(^0\) from a 10 ppb Hg\(^{2+}/\)Sn\(^{2+}\) solution and to determine the effect of Se on the Hg\(^0\) release (Fig. 9). Evolution of Hg\(^0\) at a flow rate of 50 ml min\(^{-1}\) shows an initial spike corresponding to release of Hg\(^0\), followed by an exponential decay over time \(r^2 = 0.98\). The addition of biogenic Se\(^0\) to the inline membrane filter drastically decreased initial Hg\(^0\)
Fig. 8. Characterisation of the Se particles. (A) Crystalline Se\(^0\) formed following crystallisation of abiotic Se\(^0\); (B) higher magnification view of hexagonal abiotic Se\(^0\) crystals; (C) biologically precipitated Se\(^0\) following aging under the same conditions as (A); (D) EDS of biological (i) and abiotic (ii) Se\(^0\) precipitates showing high purity following washing; (E) size distribution of biogenic (solid) and abiotic (dashed) Se\(^0\) precipitates (abiotic data from Johnson et al. [13]); (F) XRD data for initial Se\(^0\) materials (i), amorphous biogenic Se\(^0\) after 58 days, hexagonal crystalline abiotic Se\(^0\) after 53 days (iii).
emission (a decrease of 78% in peak intensity), and shows an overall decrease of 47% in Hg emission in the first 40 min. The extent of Hg capture decreases over time until Hg levels approach those of the system without biogenic Se_0.

Experiments comparing biogenic and abiotic Se_0-containing membrane filters at varying gas flow rates and reaction times using a 10 ppm Hg source show a strong correlation between the Hg sequestered and flow rate (Fig. 10), with a maximum Hg_0 sequestration of 3.4 mg m\(^{-2}\) for biogenic Se_0 at 10 ml min\(^{-1}\), nearly quadruple that of Hg_0 sequestered at a flow rate of 50 ml min\(^{-1}\). Abiotic Se_0 tested under the same conditions was capable of sequestering up to 7.7 mg Hg_0 m\(^{-2}\), double the capacity of biogenic Se_0.

Stock solutions of abiotic α-Se_0 were seen to change colour from red to black. XRD analysis showed the crystallisation of amorphous α-Se_0 Se_0 to hexagonal Se_0 (ICDD PDF Card No. 00-006-0362), whereas biological Se_0 remained stable in suspension for over a year following synthesis (Fig. 8F). Similarly, a colour change was noted in the abiotic Se_0 impregnated membrane filters exposed to 10 ppm Hg, and subsequent SEM analyses (Fig. 8A and B) show that crystallisation of the dried Se_0 powders had occurred. EDS analysis confirmed that the crystalline Se was pure Se with no Hg, indicating recrystallisation of Se rather than neocrystallisation of an HgSe phase (Fig. 8D). Biologically precipitated α-Se_0 on the impregnated filters did not change (Fig. 8C).

Johnson et al. [13] highlighted the effect of the protein bovine serum albumin (BSA) on Se_0 powders; Hg_0 capture was hindered relative to Se_0 synthesised without BSA, despite a large increase in the available surface area. This was attributed to surface passivation by BSA, reducing the density of available reactive sites on the Se_0 particle surface. Results published by Pearce et al. [14] demonstrate the presence of surface-associated proteins on Se_0 produced by G. sulfurreducens, and work published by Prakash et al. [27] show that efforts to remove the biological coatings on bacterially precipitated Se_0 resulted in the formation of similar hexagonal form seen for abiotic Se_0 (Fig. 8A). Similar organic coatings have also recently been characterised by C_60 TOF-SIMS on other complimentary bio-nanomaterials synthesised by G. sulfurreducens [28]. It is likely this surface-bound bacterially derived organic layer is increasing the

3.3.3. Sealed environment experiments

Biogenic α-Se_0 was exposed to a range of HgCl_2 concentrations between 10 ppb and 10 ppm with SnCl_2 at a 1:100 Hg:Sn ratio, and an increasing trend of Hg_0 absorption was noted (Fig. 10C), suggesting that the saturation limit of the biogenic Se_0-containing membrane filters was not reached. The maximum Hg_0 absorption of 2.2 mg m\(^{-2}\) (equivalent to 2.1 Hg by mass of Se), occurred at 10 ppm HgCl_2 and represented only 10% of the maximum Hg/Se mass ratio found by Johnson et al. [13]. Abiotic Se_0-containing membrane filters were able to capture >4 mg Hg_0 m\(^{-2}\), double the capacity of biogenic Se_0.

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stability of the Se$_0$ particles and passivating the surface, thus reducing Hg$_0$ sequestration [14,18,27].

Despite the 50% reduction in initial Hg$_0$ sequestration capacity, the long-term instability of the abiotic Se$_0$ may render this material unsuitable for application in long-term capture of Hg$_0$ emission from museum specimens. Also, as bacterially precipitated Se$_0$ is a by-product of biological selenium treatment to remove selenium from contaminated natural waters and anthropogenic waste streams [15], the more stable bacterially precipitated Se$_0$ was used for further in situ experimentation to potentially link bioremediation strategies with production of functional biomineralisers.

3.3.4. In situ experiments

Biogenic Se$_0$-containing membrane filters were placed in five boxes of the European collection with Hg$_0$ concentrations greater than 25 μg m$^{-3}$ (Fig. 2) and left for 4 weeks. The effect of the biogenic Se$_0$-containing membrane filters on the concentration of Hg$_0$ in the air within herbarium specimen boxes could not be determined due to the changes in ambient air temperature during the course of the in situ experiment; a temperature increase from 19°C to 24°C occurred during the 4 weeks, leading to an increase in Hg$_0$ release as previously observed by Oyarzun et al. [8]. An increase from 42 μg m$^{-3}$ Hg$_0$ to 90 μg m$^{-3}$ in one specimen box was seen. SEM images of the biogenic Se$_0$ before and after exposure to Hg$_0$ in the specimen boxes are shown in Fig. 7. The initial form of the α-Se$_0$ is as clearly defined spheres with smooth surfaces with particle sizes ranging from 35 to 680 nm (Figs. 7A and 8E). Following 4 weeks in Hg contaminated herbarium boxes, there was a morphological change of some spheres to a rough, textured surface (Fig. 7B). EDS spectra of remaining smooth and rough spheres showed that smooth particles contained little Hg, whereas the rough textured particles show a significant Hg concentration (Fig. 7Di). EDS analysis showed that these particles were composed of Hg and Se (31–33% Hg by mass). As pure HgSe is ~72% Hg by mass, these results suggest that there is a significant surface coating of an HgSe phase.

Following exposure of biogenic Se$_0$ to Hg$_0$ in the specimen boxes, a sample was analysed using XAS at the Hg L$_3$-edge (Fig. 6C). Linear combination fitting of the recorded XANES profiles against published standards suggests the presence of HgSe, concurrent with EDS findings. An IPD value of 7.47 eV was determined from the 1st derivation of the XANES spectra, which differs from published HgSe IPD values [23], likely as a result of the low Hg concentrations in the sample, resulting in a high signal:noise ratio (Fig. 6C, inset).

Acid digestion of the biogenic Se$_0$-containing membrane filters in aqua regia was carried out to determine total Hg concentrations (Fig. 11). All membrane filters tested showed Hg concentrations in the range 0.02–0.19 μg Hg per μg Se, corresponding to the sequestration of up to 19% Hg by mass, which is comparable to the sequestration results obtained for abiotic Se$_0$ by Johnson et al. [13].

The normalised concentration of Hg$_0$ sequestered by the biogenic Se$_0$-containing membrane filters shows a linear correlation with the Hg$_0$ concentration of the air within the herbarium specimen boxes.

4. Conclusion

Measurement of Hg concentrations in the Manchester museum herbarium has identified the presence of Hg$_0$ both within well-sealed specimen boxes and in the air of the herbarium workspaces. The concentration of Hg$_0$ in workspaces did not exceed 1.7 μg m$^{-3}$, which is well below the lowest advised workplace mercury exposure limit of 25 μg m$^{-3}$. The Hg$_0$ content observed within specimen boxes was variable and sometimes very high, increasing from 43 μg m$^{-3}$ at 19°C to 90 μg m$^{-3}$ at 24°C in one box. The construction of the specimen boxes is an advantage as it prohibits the build up of significant concentrations of Hg$_0$ within boxes, which is released when materials are accessed. Hg-contamination of specimens is variable between collections, within collections and even within specimens stored in the same box. Hg is found on specimens predominantly as metacinnabar. However, indications of a surface coating of HgO and the presence of HgCl$_2$ suggest that the form of Hg in this system is affected by a number of factors including biological activity, oxidation and original Hg concentration. Significant quantities of Hg (2.5 wt%) were found in some specimens. Hg$_0$ was not found to be directly associated with the specimens, but a strong correlation between temperature and release of Hg$_0$ was observed.

Biogenic α-Se$_0$ efficiently sequesters Hg$_0$, with a 47% initial reduction in Hg emission. Contact time between the Hg$_0$ and the biogenic Se$_0$ nanoparticles has a significant effect on the reaction rate, with the biogenic Se$_0$ capturing Hg$_0$ more effectively at lower gas flow rates, similar to the conditions of low level, continuous Hg$_0$ release expected in the sealed specimen boxes. The sequestration of the Hg$_0$ as a stable layer of HgSe on the surface of the nanoparticles represents a safe option to limit the release of Hg$_0$ into the air. The observed absorption capacity of up to 20% Hg by mass compares favourably with that observed for abiotically synthesised α-Se$_0$. A major advantage of biogenic α-Se$_0$ over abiotic α-Se$_0$ is the improvement in long term stability, which offsets the initial low reaction rates with improved longer term performance. Amorphous elemental red Se nanospheres produced both biogenically and abiotically represent a promising new way to capture Hg$_0$ released from Hg-contaminated herbarium specimens. The potential to use biogenic Se nanospheres, formed as a by-product of biological treatment of selenium contaminated wastewater, also provides an opportunity to link bioremediation strategies with production of new nanomaterials for environmental protection.

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References


Chapter 7

Research paper 4

Investigating different mechanisms for biogenic selenite transformations: *Geobacter sulfurreducens, Shewanella oneidensis* and *Veillonella atypica*

Investigating different mechanisms for biogenic selenite transformations: *Geobacter sulfurreducens*, *Shewanella oneidensis* and *Veillonella atypica*


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The metal-reducing bacteria *Geobacter sulfurreducens*, *Shewanella oneidensis* and *Veillonella atypica*, use different mechanisms to transform toxic, bioavailable sodium selenite to less toxic, non-mobile elemental selenium and then to selenide in anaerobic environments, offering the potential for in situ and ex situ bioremediation of contaminated soils, sediments, industrial effluents, and agricultural drainage waters. The products of these reductive transformations depend on both the organism involved and the reduction conditions employed, in terms of electron donor and exogenous extracellular redox mediator. The intermediary phase involves the precipitation of elemental selenium nanospheres and the potential role of proteins in the formation of these structures is discussed. The bionanomineral phases produced during these transformations, including both elemental selenium nanospheres and metal selenide nanoparticles, have catalytic, semiconducting and light-emitting properties, which may have unique applications in the realm of nanophotonics. This research offers the potential to combine remediation of contaminants with the development of environmentally friendly manufacturing pathways for novel bionanominerals.

Keywords: bionanominerals; bioreduction; selenium; metal-reducing bacteria; microbial biogeochemistry

1. Introduction

Selenium occurs in one of four oxidation states, namely the oxyanions selenate [Se(VI), \(\text{SeO}_4^{2-}\)] and selenite [Se(IV), \(\text{SeO}_3^{2-}\)], in elemental form [Se(0)] and as selenide [Se(-II)]; the latter is found in chalcogen selenides such as FeSe, in methylated compounds such as dimethylselenide and in the chalcogen-bearing proteins, selenocysteine and selenomethionine [1–4]. Elemental Se can exist in a range of structures either as \(\alpha\)-, \(\beta\)- and \(\gamma\)-monoclinic red Se comprising \(\text{Se}_8\) rings, as trigonal trapezohedral (hexagonal) black Se comprised of ordered helical [-Se-] chains, or as amorphous Se [5].

Se is found in pyritiferous and/or organic-rich lithologies and it can be released into the environment through the weathering of these materials. Seleniferous rocks, containing in excess of 100 ppm Se, outcrop in the United States, Ireland, India and China, and are often accompanied by occurrences of Se toxicity in wildfowl, cattle and the local population [6–11].

Bioavailable Se is present in the environment at very low concentrations in the form of selenate and selenite, but it can reach toxic micromolar concentrations through a combination of natural environmental processes and subsequent human activity [12]. Se pollution is also associated directly with waste materials from a broad spectrum of anthropogenic operations, including mining, agricultural, petrochemical, and industrial manufacturing operations. The problems associated with Se pollution in the aquatic environment are exacerbated through agricultural irrigation practices, particularly in arid/semi-arid regions. Shallow subsurface layers of clay impede the downward movement of irrigation water, resulting in the subsequent build up of soil trace elements as excess water evaporates from the soil surface [13]. The application of irrigation water can also solubilize and leach out naturally occurring Se in the soil, in the alkaline, oxidizing conditions prevalent in arid climates. Se present in the contaminated surface water can then enter the aquatic food chain and is bioaccumulated, in the form of proteinaceous Se, to toxic levels, particularly in fish and rice. High concentrations of bioaccumulated Se lead to deformity and death in livestock and wild fowl.

In humans, Se has the narrowest range between nutritional requirements and toxicity of any essential element [14], and thus chronic toxicity is not readily predictable, with effects of Se deficiency leading to...
cancer, severe skin lesions, heart and liver failure, while toxicity can lead to serious hair and nail disorders, gastrointestinal symptoms, ulceration and a variety of nervous disorders [15–17]. Deficiency is relatively easy to treat using dietary supplements, but an increasing recognition of Se toxicity has led to the emergence of Se as an important environmental contaminant, with the increasing use of manufactured Se nanoparticles presenting an as yet undefined risk.

Microbial processes in the environment result in Se transformations, including changes in valence or chemical form [18]. These processes can be used to develop stabilization strategies, in which toxic, bioavailable Se oxyanions are reduced to less available elemental Se. An important research area is the elucidation of the factors that influence the formation of Se nanoparticles under different conditions. Se-reducing bacteria often form nanoparticles, which can be used to develop novel Se-stabilization strategies, in which toxic, bioavailable Se is transformed into less available elemental Se. The products of these reductive transformations include a range of functional bionanominerals with interesting physical properties of potential technological significance [19–22]. In this study, different mechanisms for the reduction of Se(IV) to Se(0) are assessed in terms of the effect of alternative electron donors and the addition of an exogenous extracellular redox mediator, anthraquinone disulfonic acid (AQDS). Se is readily metabolized by microbes, and is involved in a range of metabolic processes including assimilation, methylation, detoxification, and anaerobic respiration [2,23]. For example, in Bacillus selenitireducens and Shewanella oneidensis, the reduction mechanism involves energy conservation by oxidation of lactate coupled to growth via respiratory reduction of Se(IV) using Se-specific dissimilatory enzymes [24]. Fe(III) reducers such as S. oneidensis [25] and G. sulfurreducens can also reduce Se(IV), with c-type cytochromes implicated in electron transfer to the metalloids. Unlike Shewanella and Geobacter species, V. atypica is not reported to possess high concentrations of cytochromes [26] and is thought to produce Se(0) nanospheres from Se(IV) via a hydrogenase-coupled reduction, mediated by ferredoxin. This research shows that the reduction rate and the nature of the bionanomineral phases produced can be controlled by changing reduction conditions and by selecting bacteria with particular attributes. The Se bionanomineral phases are composite materials in which biomolecules such as proteins and/or polysaccharides act as a template to direct nanoparticle nucleation and growth [27]. Identifying microbial proteins involved in environmental nanoscale bionanomineralization processes will shed light on the form and fate of aqueous and nanoparticulate metals/metalloids in natural waters and sediments [28], and will also offer the potential for synthesizing specific peptide sequences to template nanomaterials for technological and environmental applications. In this study, specific proteins implicated in playing a role in the formation of Se(0) nanospheres by G. sulfurreducens have been identified.

2. Experimental details

All chemicals were of analytical grade and obtained from Sigma-Aldrich (Dorset, UK), unless otherwise stated. All organisms were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).

2.1. Selenite reduction experiments with resting cells of G. sulfurreducens, S. oneidensis MR-1 and V. atypica

Late-exponential-phase cultures of (i) G. sulfurreducens (ATCC 51573), grown anaerobically at 30°C in defined medium with 25 mmol. l⁻¹ sodium acetate and 40 mmol. l⁻¹ sodium fumarate as the electron donor and acceptor, respectively [29,30]; (ii) S. oneidensis MR-1 (ATCC 70050) grown anaerobically at 30°C in tryptone soy broth; and (iii) V. atypica (ATCC 14894) grown anaerobically in defined medium by fermentation of 50 mmol. l⁻¹ lactate [20,31] were isolated by centrifugation (4000 × g for 20 min). The cells were washed and re-suspended in anaerobic 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (20 mmol. l⁻¹, pH 7.5) to an OD₆₀₀nm of ~1.0. These ‘resting’ cells were then challenged with Se(IV) as Na₂SeO₄ (6.5 mmol. l⁻¹) in MOPS buffer (20 mmol. l⁻¹, pH7.5) with a total volume of 20 ml. To determine the effect of an exogenous extracellular electron shuttle on Se(IV) reduction, anthraquinone-2, 6-disulphonate (disodium salt, AQDS) was added at a final concentration of 100 µM. When sodium acetate was used as a potential electron donor for reduction by G. sulfurreducens, it was added at a final concentration of 10 mmol. l⁻¹. The bottles were sealed with butyl rubber stoppers and de-gassed with N₂. When hydrogen was used as a potential electron donor, the headspace was then filled with hydrogen. The bottles containing G. sulfurreducens and S. oneidensis cultures were placed in a 30°C incubator, and the bottles containing V. atypica cultures were placed in a 37°C incubator, without shaking, for 500 h. Samples were removed for protein analysis using a bicinchoninic acid assay kit (Sigma), as described by the method of Smith et al. [32]. In order to calculate the amount of biomass from the protein analysis, it was assumed that the biomass contained approximately 55% protein by dry weight [33]. All experiments were done at least twice, in triplicate, to ensure reproducibility, and representative data are presented in this study.
2.2. Ion chromatography (IC) and inductively coupled plasma–atomic emission spectrometry (ICP–AES)

Aliquots were removed from experimental cultures under an anaerobic atmosphere, using a sterile needle, passed through a 0.22 μm filter and flash frozen in liquid nitrogen for storage at −80°C. The total SeO$_3^{2−}$ concentration was determined by IC ( Dionex, UK) and the total Se concentration in solution by ICP–AES (Horizon, V.G. elemental, UK). Se(0) was calculated by subtracting total Se in solution from the total Se starting concentration (6.5 mmol. l$^{-1}$) and Se(-II) was calculated by subtracting concentration of Se(IV) in solution from total Se in solution. ICP–AES was carried out under an anaerobic atmosphere to prevent oxidation of reduced Se species. For IC, a Dionex DX600 system with GP50 gradient pump and CD20 conductivity detector was used, with an injection loop of 50 μ L and wide-bore (4 mm) columns. The mobile phase was isocratic 3.6 mmol. l$^{-1}$ Na$_2$CO$_3$/3.4 mmol. l$^{-1}$ NaHCO$_3$ with a flow rate of 1.4 mL/min. The system backpressure was 2600–2800 psi. For ICP–AES analysis of Se a wavelength of 196 nm was used. For both techniques, calibration standards of 0, 12.5, 50 and 100 mmol. l$^{-1}$ Na$_2$SeO$_3$ were prepared by dilution of concentrated reference element stock solutions. Calibration blocks were placed at intervals throughout each analytical run in order to correct for instrument drift. The average analytical error was <3% for IC data and ±0.103 (3 × σ) for ICP–AES. Figures 1 to 4 show the initial change in concentration with time and a measurement after reduction for 500 h in the presence of the organisms is quoted as the end point in the text.

2.3. X-ray absorption spectroscopy (XAS)

XAS data were collected at the STFC Daresbury SRS synchrotron, on Wiggler beam line 16.5, with the storage ring operating at 2 GeV and a current of 100–200 mA. A pre-monochromator 1.2 m uncoated mirror was used, bent to provide vertical collimation, and to remove some higher energy photons. A Si(220) double crystal monochromator was employed, detuned to 70% transmission to minimize any residual harmonics. Energy calibration was performed using a standard sample of red elemental selenium, ground and diluted with boron nitride. Samples were measured in a 1 mm thick aluminium frame sample holder with Mylar windows at ca. 77 K using a cold-finger stage. Spectra were collected at the Se K-edge in fluorescence-yield mode over the energy range 12,400–13,300 eV, with the sample cell at 45° to the incident X-ray beam, using a Canberra 30-element solid state detector. The structural environment was determined using extended X-ray absorption fine structure (EXAFS) and X-ray absorption near edge structure (XANES) analysis. Background subtracted EXAFS spectra were analysed in EXCURV98 using full-curved-wave theory [34]. Phase shifts were derived in the programs from ab initio calculations using Hedin-Lundqvist potentials and von Barth ground states [35]. Fourier transforms of the EXAFS spectra were used to obtain an approximate radial distribution function around the central Se atom (the absorber atom); the peaks of the Fourier transform can be related to shells of surrounding back-scattering atoms characterized by atom type, number of atoms in the shell, the absorber–scatterer distance and the Debye–Waller factor, 2σ (a measure of both the thermal motion between the absorber and scatterer and of the static disorder or range of absorber–scatterer distances). The data were fitted for each sample by defining a theoretical model and comparing the calculated EXAFS spectrum with the experimental data. Shells of back-scatterers were added around the selenium and by refining an energy correction $E_F$ (the Fermi energy), the absorber–scatterer distance and Debye–Waller factor for each shell, a least squares residual (the R-factor [36]) was minimized. The coordination numbers were refined for oxygen atoms from water molecules for hydrated HSe$^-$ (hydrogen atoms are too weak as scatterers to be detected by XAS).

2.4. Transmission electron microscopy

Transmission electron microscopy (TEM) was used to examine the structure of the washed cells and accumulation of selenium minerals after exposure to Se(IV) for 500 h. Cells were collected and washed three times in anaerobic 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mmol. l$^{-1}$, pH 7), then fixed with 2.5% glutaraldehyde. Whole mounts of bacteria were placed on carbon-coated Cr 200-mesh copper grids. Samples were examined using a FEI Tecnai 12 Biotwin transmission electron microscope (FEI, Eindhoven, The Netherlands) at 100 kV. EDX was also performed at 100 kV by using a spot size of 200 nm, a current of 25 mA, and a (live) counting time of 100 s.

2.5. X-ray diffraction

X-ray diffraction (XRD) measurements were acquired over a 20 range of 20°–60° with a step size of 0.02° and a scan time of 2 s per step, using a Bruker D8 Advance X-ray diffractometer (Bruker AXS Ltd, Coventry, UK) with Cu Kα1 source. The samples were dried to form a uniform film on a glass slide, which was transferred to the instrument immediately after preparation.

2.6. Identification of proteins associated with Se(0) nanospheres produced by G. sulfurreducens

To identify proteins associated with Se(0) nanospheres produced by G. sulfurreducens, cultures
grown anaerobically at 30°C for one week, in defined medium supplemented with Na₂SeO₃ (1 mmol·L⁻¹), were homogenized by syringing through a sterile 21-gauge syringe needle and pelleted by centrifugation. The Se(0) nanospheres were fractionated from cells and media using a density-based approach involving iterative centrifugation through a chemically inert solution, which preserves any organic biomolecules by avoiding the use of surfactants or solvents [28]. The pellet was re-suspended in lithium polytungstate solution (LST) of density ∼2.8 g cm⁻³ (Geoliquids, Prospect Heights, IL, USA), and then subjected to further low-speed centrifugation (5 min at 1000 rpm) in autoclaved Eppendorf tubes. The Se(0) nanosphere fraction (ρ ∼4.8 g cm⁻³) was easily pelleted at the bottom of LST-filled tubes during centrifugation, while the cell biomass fraction rose to the top of the LST solution. The separated fractions were washed several times in deionized water to remove LST. The nanosphere-rich and nanosphere-poor fractions from the separation process were heated at 110°C for 30 min in reducing Laemmli sample buffer (2% sodium dodecyl sulphate (SDS), 3% 2-mercaptoethanol, 40 μL) to denature any proteins. The separate fractions were then centrifuged (13000 × g for 5 min) to remove the Se(0) nanospheres prior to SDS polyacrylamide gel electrophoresis. The fractions (25 μL) were run in an 18% Tris-HCl gel with Tris/glycine/SDS running buffer. Kaleidoscope (BioRad) pre-stained molecular weight ladders were run on both sides of the gel and gel-separated protein bands were detected using Imperial Protein Stain. Bands of interest were excised under sterile conditions for trypsin digestion. Tryptic peptides were analysed using liquid chromatography-based high-resolution tandem mass spectrometry (LC-MS/MS). Proteins of interest were identified by matching MS/MS spectra against a database constructed using predicted peptides from the Geobacter sulfurreducens PCA genome [37] using the SEQUEST algorithm [38] and filtered with BioWorks and DTASelect [39] at the peptide level using conservative filters [Xcorr’s of at least 1.8 (+1), 2.2 (+2) 3.5 (+3)]. Only proteins identified with two fully tryptic peptides at conservative filters were considered for further biological study.

3. Results and discussion

3.1. Mechanisms and kinetics of Se(IV) reduction

3.1.1. Se(IV) reduction by G. sulfurreducens using acetate or hydrogen as the electron donor

For G. sulfurreducens with acetate as an electron donor (Figure 1 A and B), IC showed that the Se(IV) reduction rate was increased by a factor of three as a result of the addition of AQDS (1.1 mmol·L⁻¹ Na₂SeO₃ g⁻¹ (biomass) h⁻¹ with AQDS versus 0.4 mmol·L⁻¹ Na₂SeO₃ g⁻¹ (biomass) h⁻¹ without). ICP–AES showed that this reduction in Se(IV) corresponded to a decrease in total soluble Se, presumably owing to the formation of insoluble Se(0) (noted as a red precipitate). A significant concentration (2.5 mmol·L⁻¹) of Se(IV) remained in solution after reduction by G. sulfurreducens for 500 h without AQDS, with the majority of the remaining Se present as Se(0), and a minor proportion (∼15%) reduced to the soluble Se(-II) phase. In the absence of AQDS, no Se(IV) remained after 500 h reduction and Se(0) was the dominant phase, with approximately a third of the total Se further reduced to Se(-II), as indicated by the concentration of total Se in solution from ICP–AES. There was a slight decrease in the amount of Se(0) corresponding to formation of Se(-II) after 16 h, even when a significant concentration of Se(IV) remained in solution, suggesting that the reduction mechanism was not biphasic and that Se(0) was reduced continuously to Se(-II) (Figure 1 B). Figure 1 C shows the formation of Se(0) nanospheres (50–100 nm) on the surface of the rod-shaped G. sulfurreducens cells after 24 h reduction and is representative of samples both with and without AQDS. This spherical morphology has been reported previously for extracellular Se precipitates [22,25,27]. Figures 1 D and E show the Se(0) nanophases formed after 500 h reduction without and with AQDS, respectively. The composition of the Se(0) nanophases produced during the reduction of Se(IV) by G. sulfurreducens was confirmed by EDX and a representative spectrum of the particles in Figure 1 C to E is provided in Figure 1 F. A large difference in the size and morphology of the particles can be observed between Figures 1 D and E, highlighting the significance of the redox mediator in the reduction mechanism. Without AQDS, the end product was predominantly red amorphous Se(0), comprising disordered [-Se-]₌ chains and Se₈-rings (Figure 1 D). Diffraction data (Figure 1 G), obtained only from the acicular structures of 1–2μM in length, visible in Figure 1 D, show weak Bragg peaks at 23.5 and 29.7, corresponding to the (100) and (101) reflections of Se(0), respectively [5]. It has been reported that amorphous Se(0) is unstable at ambient temperatures, and in heating experiments undergoes a glass transition in the range 30–47°C, crystallization (to the trigonal form) in the range 120–150°C and melting ∼222°C [40,41]; the activation energy for the crystallization is 40–60 kJ/mol [42]. Under the conditions described here, the red amorphous phase was still present after several months, suggesting that organic material associated with the G. sulfurreducens cells may play a role in stabilizing the Se(0) nanospheres. Kessi et al. [27] also reported that the amorphous Se allotrope produced by Rhodospirillum rubrum was stable for
Figure 1. Graphs to show reduction of Se(IV) (♦) and the formation of Se(0) (■) followed by Se(-II) (▲) using *G. sulfurreducens* with acetate, without AQDS (A) and with AQDS (B). TEM (whole mount) of *G. sulfurreducens* showing Se precipitates after 24 h Se(IV) reduction (C) and after 500 h Se(IV) reduction without AQDS (D) and with AQDS (E). EDX of Se precipitates (F). X-ray powder diffraction data for Se precipitates after 500 h Se(IV) reduction (G).
months in the presence of the cultures. The Se(0) produced in the presence of AQDS was predominantly in the form of stubby trigonal crystallites comprising ordered helical \([-\text{Se}_2\] \) chains linked together by inter-chain van der Waals forces [43], as confirmed by the strong, sharp (100) and (101) reflections in Figure 1 G.

Reduction of Se(IV) by \(G.\ sulfurreducens\) using hydrogen as the electron donor occurs at a faster rate than that observed with acetate (Figure 2). Again, the reduction rate was improved by the addition of AQDS (1.1 mmol. \(\text{Na}_2\text{SeO}_3\) \(g^{-1}\) (biomass) \(h^{-1}\) with AQDS versus 0.8 mmol. \(\text{Na}_2\text{SeO}_3\) \(g^{-1}\) (biomass) \(h^{-1}\) without), as indicated by IC. ICP–AES showed that reduction of Se(IV) corresponded to a decrease in total soluble Se, attributable presumably to the rapid formation of red insoluble Se(0), confirmed visually (Figures 2 A and B). No detectable Se(IV) was present in solution after reduction for 500 h both with and without AQDS. The dominant Se phase after 500 h reduction was soluble Se(-II), as confirmed by XAS and total Se in solution measured using ICP–AES, with 4.5 mmol. \(1^{-1}\) and 4.0 mmol. \(1^{-1}\) present in the solutions with and without AQDS, respectively. Again, the presence of both Se(IV) and Se(-II) at the same time is indicative of a continuous reduction mechanism from Se(IV) to Se(-II) (Figures 2 A and B). Formation of Se(0) nanospheres (50–100 nm) on the surface of the \(G.\ sulfurreducens\) cells after 24 h reduction is shown in Figure 2 C. Figure 2 D shows bright field and dark field TEM images of the stubby trigonal Se(0) crystallites formed after 500 h reduction, and is representative of the samples both with and without AQDS. The composition of the Se(0) nanophases produced during the reduction of Se(IV) by \(G.\ sulfurreducens\) was confirmed by EDX and a representative spectrum of the particles in Figures 2 C and D is provided in Figure 2 E. The crystal structure of the Se(0) after 500 h was confirmed by the diffraction data shown in Figure 2 F. The shape and size of crystallites formed by \(G.\ sulfurreducens\) using hydrogen as electron donor (Figure 2 D) using acetate as the electron donor with AQDS (Figure 1 E) were very similar, but much less precipitated material was present in the samples with hydrogen owing to further reduction to the soluble Se(-II) phase. The soluble Se-bearing phase was analysed using XAS at the Se K-edge (Figures 2 G, H and I). The magnitude of the EXAFS oscillations (Figure 2 G) was very small indicating that Se was surrounded by weak scatterers. The best fit for the Fourier transform (Figure 2 H) gave no clear indication of any neighbouring atoms closer than five oxygens at 3.34 Å, suggesting that the selenium had completely dissociated and was surrounded by water molecules (Table 1). This long distance reflects the attraction of the \(\delta^+\) hydrogen atoms towards the negatively charged Se(-II), so that the water molecules are orientated with the oxygens further away.

Hydrogen is a very weak scatterer and is only detectable by EXAFS in high-concentration solutions with very well-ordered water molecules around the dissolved ion [44]. The XANES (Figure 2 I) is distinct from that for Se(VI), Se(IV) or Se(0) and the lower edge position indicates that it is a more reduced species.

### 3.1.2 Se(IV) reduction by \(S.\ oneidensis\) using hydrogen as the electron donor

\(S.\ oneidensis\) is unable to metabolize acetate as an electron donor under anaerobic conditions [45]. Thus, hydrogen was used as the sole electron donor for reduction of Se(IV). IC showed that the Se(IV) reduction rate more than doubled upon addition of AQDS (0.5 mmol. \(1^{-1}\) \(\text{Na}_2\text{SeO}_3\) \(g^{-1}\) (biomass) \(h^{-1}\) with AQDS versus 0.2 mmol. \(1^{-1}\) \(\text{Na}_2\text{SeO}_3\) \(g^{-1}\) (biomass) \(h^{-1}\) without). Although they were considerably less efficient than \(G.\ sulfurreducens\) cells, the \(S.\ oneidensis\) cells also employed a continuous mechanism to reduce Se(IV), through Se(0), down to Se(-II) (Figures 1 to 3). A significant concentration of Se(IV) (2.6 mmol. \(1^{-1}\)) remained in solution after 500 h reduction without AQDS, and overall very little Se(-II) was produced with or without AQDS. Figure 3 C shows Se(0) nanospheres on the surface of the rod-shaped \(S.\ oneidensis\) cells after 24 h reduction without AQDS. These are slightly larger nanospheres (100–250 nm) than those observed previously (Figures 1C and 2C) and those formed by \(S.\ oneidensis\) with AQDS (data not shown), which could be a result of the slower rate of reaction [46]. Klonowska et al. [25] also recorded spherical deposits of Se(0) attached to \(S.\ oneidensis\) cells and suggested that Se(IV) reduction occurs at the surface of the cell as it is related to the electron transfer capacity of cytochrome c on the outer membrane. Figure 2 D shows Se(0) nanospheres formed after 500 h reduction, and is representative of the samples both with and without AQDS. It can be seen that, after this longer period of reduction, three different sizes (~200, 100 and 50 nm) of Se(0) nanospheres are present, but it is unclear why these particular particle sizes appear to predominate. The composition of the Se(0) nanophases produced during the reduction of Se(IV) by \(S.\ oneidensis\) was confirmed by EDX and a representative spectrum of the particles in Figures 3 C and D is provided in Figure 3 E. The Se(0) nanospheres produced by \(S.\ oneidensis\) without AQDS were amorphous and no Bragg peaks were visible in the XRD data. However, with AQDS, the diffraction data (Figure 3 F) show weak Bragg peaks corresponding to the (100) and (101) reflections of Se(0). This sample was also a darker shade of red, suggesting the formation of a more (but poorly) crystalline product when AQDS was added.
Figure 2. Graphs to show reduction of Se(IV) (♦) and the formation of Se(0) (■) followed by Se(-II) (▲) using *G. sulfurreducens* with hydrogen, without AQDS (A) and with AQDS (B). TEM (whole mount) of *G. sulfurreducens* showing Se precipitates after 24 h Se(IV) reduction (C), and bright field and dark field TEM after 500 h Se(IV) reduction (D). EDX of Se precipitates (E). X-ray powder diffraction data for Se precipitates after 500 h Se(IV) reduction (F). Se K-edge (G) $k^2$-weighted EXAFS spectra (H) Fourier transforms and (I) XANES spectra of soluble Se phase produced after 500 h Se(IV) reduction using *G. sulfurreducens* with hydrogen.
Table 1. Results of XAS data analysis for the soluble Se phase.

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. and type of atom</th>
<th>Shell radius (Å)</th>
<th>Debye–Waller factor (Å²)</th>
<th>R value/data range (k)</th>
<th>Suggested phase</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. sulfurreducens</em></td>
<td>5 O</td>
<td>3.34</td>
<td>0.035</td>
<td>30.5/3–8</td>
<td>HSe⁻</td>
</tr>
<tr>
<td><em>V. atypica</em></td>
<td>4 O</td>
<td>3.32</td>
<td>0.036</td>
<td>38.2/3–8</td>
<td>HSe⁻</td>
</tr>
</tbody>
</table>

Figure 3. Graphs to show reduction of Se(IV) (♦) and the formation of Se(0) (■) followed by Se(-II) (▲) using *S. oneidensis* with hydrogen, without AQDS (A) and with AQDS (B). TEM (whole mount) of *S. oneidensis* showing Se precipitates after 24 h Se(IV) reduction without AQDS (C) and after 500 h Se(IV) reduction (D). EDX of Se precipitates (E). X-ray powder diffraction data for Se precipitates after 500 h Se(IV) reduction (F).
3.1.3 Se(IV) reduction by V. atypica using hydrogen as the electron donor

The ability of V. atypica to reduce Se(IV) was assessed with hydrogen as the sole electron donor, because acetate did not support Se(IV) reduction under anaerobic conditions [20]. Figures 4 A and B show a vast difference in the ability of this organism to reduce Se(IV) with and without AQDS. The initial Se(IV) reduction rate for V. atypica cells without AQDS was 0.2 mmol. 1\(^{-1}\) Na\(_2\)SeO\(_3\) g\(^{-1}\) (biomass) h\(^{-1}\), and the dominant phase remained as Se(IV) after 500 h reduction (3.7 mmol. l\(^{-1}\)). With AQDS, the V. atypica cells were able to reduce Se(IV) at a rate of 1.1 mmol. 1\(^{-1}\) Na\(_2\)SeO\(_3\) g\(^{-1}\) (biomass) h\(^{-1}\), five times faster than without AQDS, and the soluble Se(-II) phase accounted for 100% of the Se after 500 h reduction. Figures 4 C and D show nanospheres on the surface of the V. atypica micrococio and intracellular nanospheres respectively, after 24 h reduction with AQDS. EDX suggested that the particles were Se-rich, in keeping with formation of elemental Se (Figure 4 E). The relatively small size of the nanospheres (∼50 nm) could be a result of a faster rate of reduction [46]. After 500 h reduction without AQDS, the Se(0) precipitates remained as red nanospheres and the lack of Bragg peaks in the diffraction data confirmed that it was completely amorphous. The soluble Se(-II) phase produced after Se(IV) reduction for 500 h by V. atypica cells, with AQDS, was analysed using XAS at the Se K-edge (Figures 4 F, G and H). The weak EXAFS oscillations (Figure 4 F) were again indicative of weak scatterers surrounding the central Se atom. The best fit for the Fourier transform (Figure 4 G) indicated four oxygens at a very similar distance (3.32 Å) to that found for the Se(-II) produced by G. sulfurreducens suggesting that the selenium was present as dissociated HSe\(^-\) surrounded by water molecules (Table 1). The difference between the coordination numbers (N) in Table 1 is probably not significant as N in EXAFS can often have errors of 20%, especially in analysis of dilute solutions [47]. The XANES (Figure 4 H) is also very similar to that for the Se(-II) produced by G. sulfurreducens (Figure 2 I). It has been shown that the biogenic Se(-II) solution, produced by G. sulfurreducens and V. atypica under ambient conditions, as opposed to the hazardous, expensive production of NaHSe from Al\(_2\)Se\(_3\), can be employed in an aqueous-based, wet chemical synthesis for the fabrication of CdSe/ZnSe quantum dots [20,48].

3.1.4 Comparison of mechanisms and kinetics of Se(IV) reduction by the different organisms

The graphs showing the rate of Se(IV) reduction and the formation of Se(0) followed by Se(-II), with hydrogen and AQDS, using V. atypica (Figure 4 B), S. oneidensis (Figure 3 B) and G. sulfurreducens (Figure 2 B), suggests that these organisms may employ different reduction mechanisms. For V. atypica, the production of Se(-II) occurred only after 100% of the Se(IV) had been reduced to Se(0) and was, therefore, a biphasic reaction. This type of biphasic reduction reaction for the production of Se(-II) has been reported previously for the Se(IV)-respiring Gram-positive haloalkaliphile, B. selenitireducens [49]. For S. oneidensis cells, production of Se(-II) was limited, but for G. sulfurreducens cells, a substantial amount of Se(-II) was produced by the continuous reduction of Se(IV) through Se(0) to Se(-II). These differences may be explained by considering the way in which these organisms potentially interact with electron acceptors. Zannoni et al. [2] proposed a general biochemical mechanism for producing Se(0) in bacterial cultures, involving the reaction between Se(IV) and reduced thiols, which are regenerated by the action of glutathione reductase and/or thioredoxin reductase; all of the organisms in this study may employ this intracellular mechanism to some degree. Indeed, the annotation for the sequenced organisms lists glutathione reductase and thioredoxin reductase for S. oneidensis and glutathione reductase for G. sulfurreducens. However, G. sulfurreducens and S. oneidensis can potentially use a different mechanism, involving c-type cytochromes on the outer membrane, which are implicated in the reduction of a wide range of metals, including Fe(III) oxides [50]. S. oneidensis has also been shown to release flavins as electron-shuttling compounds [51] and, although G. sulfurreducens has not been shown to synthesize electron-shuttling compounds, both of these organisms are able to use exogenous extracellular electron shuttles, such as AQDS, to transfer electrons from the cell to extracellular electron acceptors [52]. Unlike Shewanella and Geobacter species, V. atypica is not reported to possess high concentrations of cytochromes [26] and it is proposed that the production of reduced selenium species is achieved via a hydrogenase-coupled reduction, mediated by ferredoxin [53]. It is possible that S. oneidensis and G. sulfurreducens are better equipped to deal with both the soluble Se(IV) and the insoluble Se(0) as electron acceptors in the extracellular environment, owing to their extensive array of outer membrane cytochromes [37]. The difference in the ability of V. atypica to reduce Se(IV) with and without AQDS also supports this hypothesis. Without AQDS, V. atypica may only be able to transfer electrons to Se(IV) via the intracellular ferrodoxin-mediated pathway. With a redox mediator, V. atypica has the potential to transfer reducing equivalents to extracellular Se(IV), generating both intracellular and extracellular Se(0) precipitates, which can be reduced subsequently to Se(-II).
Figure 4. Graphs to show reduction of Se(IV) (♦) and the formation of Se(0) (■) followed by Se(-II) (▲) using *V. atypica* with hydrogen, without AQDS (A) and with AQDS (B). TEM (whole mount) of *V. atypica* after 24 h Se(IV) reduction with AQDS showing extracellular Se precipitates (C), and bright field and dark field TEM showing intracellular Se precipitates (D). EDX of Se precipitates (E). Se K-edge (F) $k^2$-weighted EXAFS spectra (G) Fourier transforms and (H) XANES spectra of soluble Se phase produced after 500 h Se(IV) reduction using *V. atypica* with hydrogen.
3.2. Identification of proteins associated with Se(0) nanospheres produced by G. sulfurreducens

Kessi et al. [27] reported that a protein content of about 20 mg of protein/mmol. l⁻¹ of selenium was found in suspensions of Se(0) particles isolated from culture media after growth in the presence of Se(IV) and suggested the existence of a selenium–protein complex. However, successful identification of the proteins involved in these complexes has not, as yet, been achieved. To this end, biogenic Se(0) nanospheres produced during growth of G. sulfurreducens were isolated using a density-based separation technique. Figure 5 shows an SDS PAGE image of the nanoparticle-poor (A) and nanoparticle-rich (B) fractions obtained. The gel band (highlighted) produced by proteins associated with Se(0) nanospheres when reacted with a protein-specific stain was excised and the proteins identified by LC–MS/MS. The results suggest that the Se(0) nanoparticles produced by G. sulfurreducens were associated with a 15 kDa c-type cytochrome. Peptides were detected matching this protein with a sequence coverage of 31.5% and the location in the gel (Figure 5) matched the predicted molecular mass of the annotated protein. The c-type cytochromes have also been shown to be essential for the formation of extracellular UO₂ nanoparticles by S. oneidensis MR-13, and implicated in the reduction of a wide range of other metals [54]. This protein has not been characterized previously but analysis of the gene sequence by the SignalP program [55] suggests post-translational secretion of the cytochrome, consistent with its association with extracellular Se nanospheres.

Proteolytic fragments of the redox-active, iron–sulphur protein ferredoxin oxidoreductase were also identified in the G. sulfurreducens band, suggesting that this organism may employ an intracellular ferredoxin-mediated pathway for the reduction of Se(IV) similar to that observed for V. atypica, followed by secretion or release of the Se nanospheres through cell lysis.

4. Conclusions

Anaerobic bioreactor systems have been shown to efficiently remove Se oxyanions from aqueous waste streams [56]. When evaluating Se biotreatment systems, it is essential to study the underlying biological mechanisms of reduction. The reduction rate and the nature of the selenium phases produced is strongly influenced by the nature of the organisms and by the reducing conditions employed. While this may present problems in terms of the formation of a dissolved Se(-II) species, rather than insoluble Se(0) that can be retained in the bioreactor, it does offer the potential to link bioremediation strategies with production of functional bionanomaterials. The products of Se(IV) biotransformations include: (i) red amorphous Se(0) nanospheres which act as an efficient sorbents for problematic vaporous contaminants, such as mercury [57], as well as having useful photo-optical and semiconducting properties for application in photocopiers, microelectronic circuits and solar cells [22]; (ii) black crystalline trigonal Se(0) which is a p-type extrinsic semiconductor and is used in producing photocells, photographic exposure meters, pressure sensors and electrical rectifiers because of its high photoconductivity [58]; and (iii) Se(-II), which can be used in the synthesis of fluorescent, semiconductor CdSe/ZnSe quantum dots with applications in biomedicine [59], electronics [60] and chemoselective photocatalysis [61].

It is also important to consider the potential impact of microbial processes on Se speciation in sediments. For example, in sediments dominated by Geobacter species, Se may well be fully reduced to the Se(-II) form where it could accumulate as FeSe [49], particularly if humics are present to act as redox mediators, whereas Se(0) would potentially be the major sink for Se oxyanions in sediments dominated by Shewanella species. However, careful examination of the total Se budget in sediment systems, including the impact of competing bio-methylation reactions [62], is required to fully assess the impact of different microbial communities.

This research represents the first attempt to identify the proteins present in protein–nanoparticle complexes, with the discovery that c-type cytochromes and ferredoxin are associated with the post reduction mineral
phases and may therefore be involved in the formation of Se(0) nanopospheres by G. sulfurreducens. These protein–nanoparticle complexes could play a key role in biomineral synthesis by bacteria and a systematic study of the role of microbial proteins in nanoscale biomineralization processes will provide a valuable insight into the fate of nanoparticulate metals/metalloids in natural waters, as well as potentially facilitating the discovery of specific peptide sequences to template nanomaterials for technological applications.

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References


Chapter 8

Research paper 5

Ex situ formation of metal selenide quantum dots using bacterially derived selenide precursor

Ex situ formation of metal selenide quantum dots using bacterially derived selenide precursors

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Abstract

Biologically synthesised Se II is assessed for suitability as a starting material for quantum dot formation. Biogenic Se II is produced by the reduction of Se IV by Veillonella atypica and compared directly against borohydride-reduced Se IV in the production of glutathione stabilised CdSe and β-mercaptoethanol stabilised ZnSe nanoparticles by aqueous synthesis. Findings show biological Se II forms smaller, narrower size distributed QDs under the same conditions and growth kinetics show slower growth of biologically sourced CdSe phases. Proteins isolated from sterilised biogenic Se II have been identified as belonging to the closely related V. parvula. XAS analysis of the S K-edge indicates glutathione-sourced S is structurally incorporated within CdSe. A novel synchrotron based XAS technique was developed to follow the nucleation of biological and inorganic selenide phases, and biogenic Se II displayed a higher tolerance to beam-induced oxidative damage and suggested slower initial reaction rates. The bacterial production of quantum dot precursors offers an alternative, ‘green’ synthesis technique that negates the requirement of expensive, toxic chemicals.
and suggests a possible link to the exploitation of selenium contaminated waste streams.

**Keywords:** quantum dots, chalcogenide, cadmium selenide, bionanomaterials, *Veillonella atypica.*

1. **Introduction**

The involvement of microorganisms in the synthesis of novel, nanocrystalline particles represents a low cost, environmentally-friendly method of producing industrially and technologically relevant nanomaterials. Microorganisms have been used to synthesise a range of nanomaterials including Fe oxides, precious metal catalysts and a suite of nanocrystalline, semiconducting ‘quantum dots’ (6, 23, 24).

The interest in quantum dots (QDs) is due to their unique semiconducting properties induced by the onset of quantum confinement in the size range 1-20nm, generating optical, electrical and mechanical properties that differ from those of the bulk materials (35). The photooptical and photovoltaic properties of the II/VI semiconductors (metal chalcogenides) are particularly suited for their application in solar cells and optoelectronic sensors (5, 13, 35) as well as fluorescent biolabelling, including their role in cancer screening (14, 16, 38).

Of all QDs synthesised, cadmium-based QDs (as CdS, CdSe and CdTe) are the most thoroughly explored owing to fluorescent emissions across the visible spectrum by direct manipulation of particle size. However, the presence of highly toxic Cd and Te, and to a lesser extent Se, is a barrier to the development of wider applications. Recent trends have seen the development of cadmium-free QDs, including the investigations of alternative metals such as zinc, and the use of capping agents to limit toxic effects (15, 29, 32, 39).

Traditional organochemical synthesis of chalcogenide quantum dots involves the injection of reactants into heated organic solvents, commonly tri-n-octylphosphine oxide (TOPO), and requires the use of a number of toxic,
expensive precursors and necessitates the exchange of surfactant molecules to aid aqueous solubility (4, 11). Simpler, aqueous-based synthesis techniques have subsequently been developed in which quantum dots are synthesised in a highly repeatable, single-step reaction at temperatures <100°C, using surfactant molecules to increase water solubility, stability and limit particle growth (4, 12, 13). Thiol compounds have been identified as ideal surfactants, and investigations by Rogach et al (31) and Gaponik et al (13) identify thioalcohols such as β-mercaptoethanol as especially suited to minimising the size of initial precipitates. Biologically derived sulphurous materials such as the peptides glutathione (GSH) and bovine serum albumin (BSA) have also been successfully used in the aqueous synthesis of metal chalcogenide quantum dots (4, 8, 10, 25, 28, 36, 40, 41).

The whole-cell biological synthesis of II/VI quantum dots has been reported as a greener, environmentally-friendly alternative to chemical synthesis. The use of a number of fungal (2, 7, 18-20) and bacterial (1, 3, 28, 33, 34) species in the formation of a range of quantum dots has been demonstrated (table 1), and typically exploits heavy metal detoxification mechanisms; Cd^{2+} detoxification by yeast species including *Schizosaccharomyces pombe*, *Candida glabrata* (8, 10) and *Saccharomyces cerevisiae* (2) stimulates the secretion of extracellular sulphurous proteins which act as surfactants as well as stimulating sulphide generation, resulting in extracellular deposition of peptide-coated fluorescent CdS nanocrystals. The use of oxyanions of the chalcogens Se and Te as precursors typically requires the addition of a strong reducing agent such as sodium borohydride to produce the required Se^{2-} or Te^{2-} anions (2, 3, 6, 36, 37, 41), however, Pearce et al (27) demonstrate the ability of the clinical isolate *Veillonella atypica* to reduce aqueous Se^{IV} to Se^{2-} through a biphasic reduction pathway, suggesting the possibility of linking the biosynthesis of quantum dot precursors to the bioremediation of selenium contaminated waste streams (22). Further research on this bacterium demonstrated the feasibility of the use of biologically prepared aqueous Se^{2-} solutions in the formation of metal selenide quantum dots (28).
<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Microorganism</th>
<th>Product</th>
<th>Metal Reactant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fungi</strong></td>
<td><em>Fusarium oxysporum</em></td>
<td>CdSe</td>
<td>CdCl₂</td>
<td>Kumar <em>et al</em> (20)</td>
</tr>
<tr>
<td></td>
<td><em>Candida glabrata</em></td>
<td>CdS</td>
<td>CdSO₄</td>
<td>Dameron <em>et al</em> (7)</td>
</tr>
<tr>
<td></td>
<td><em>Schizosaccharomyces pombe</em></td>
<td>CdS</td>
<td>CdSO₄</td>
<td>Dameron <em>et al</em> (7), Kowshik <em>et al</em> (18)</td>
</tr>
<tr>
<td></td>
<td><em>Torulopsis sp.</em></td>
<td>PbS</td>
<td>Pb(NO₃)₂</td>
<td>Kowshik <em>et al</em> (19)</td>
</tr>
<tr>
<td></td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>CdTe</td>
<td>CdCl₂</td>
<td>Bao <em>et al</em> (2)</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td><em>Escherichia coli</em></td>
<td>CdS</td>
<td>CdCl₂</td>
<td>Sweeney <em>et al</em> (34)</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
<td>CdTe</td>
<td>CdCl₂</td>
<td>Bao <em>et al</em> (3)</td>
</tr>
<tr>
<td></td>
<td><em>Klebsiella pneumoniae</em></td>
<td>CdS</td>
<td>Cd(NO₃)₂</td>
<td>Smith <em>et al</em> (33)</td>
</tr>
<tr>
<td></td>
<td><em>Rhodobacter sphaeroides</em></td>
<td>ZnS</td>
<td>ZnSO₄</td>
<td>Bai <em>et al</em> (1)</td>
</tr>
<tr>
<td></td>
<td><em>Veillonella atypica</em></td>
<td>CdSe</td>
<td>Cd(ClO₄)₂</td>
<td>Pearce <em>et al</em> (28)</td>
</tr>
</tbody>
</table>

*Table 1.* A number of fungal and bacterial species have been used in the synthesis of a number of metal chalcogenide nanoparticles.

This research reports the *ex situ* formation and characterisation of metal selenide quantum dots using bacterially generated Se⁺⁺ as an alternative to chemically synthesised precursors. Biogenic and abiotic fluorescent nanoparticles were characterised and compared using UV/Vis
spectrophotometry, photoluminescence spectroscopy and transmission electron microscopy. Bacterial proteins present within filter sterilised biogenic Se\textsuperscript{II} solutions were identified by polyacrylamide gel electrophoresis. The chemical interaction between the nanoparticles and glutathione was explored using S K-edge synchrotron x-ray absorption spectroscopy (XAS). Finally, this work investigates the effects of extracellular biological materials on the stability, nucleation and growth of β-mercaptoethanol stabilised, cadmium-free zinc selenide nanoparticles using a novel \textit{in situ} time resolved synchrotron XAS technique.

2. Methods

All chemicals used in this work were of analytical grade and acquired from Sigma-Aldrich, UK.

2.1 Bacterial synthesis of Se\textsuperscript{II}.

\textit{Veillonella atypica} (ATCC 14894) was grown anaerobically in defined media, coupling the reduction of 1.0g l\textsuperscript{-1} glucose to the oxidation of 7.5g l\textsuperscript{-1} sodium lactate (28). Cells were isolated by centrifugation and resuspended into sterile, anaerobic 20mM 3-(N-morpholino)propanesulphonic acid (MOPS) buffer at pH7.5 to an OD\textsubscript{600} of \textasciitilde1.0. Cultures were amended with 5mM Na\textsubscript{2}SeO\textsubscript{3} and 100\textmu M of the electron shuttling compound anthraquinone-2,6-disulphonate (AQDS). The headspaces of the sealed culture bottles were replaced with H\textsubscript{2} to serve as the electron source. Se\textsuperscript{IV} amended cultures were then incubated at 37\textdegree C for 72 hours, by which time a colour change from the initial clear, colourless solution to a clear, yellow solution had occurred, via an intermediate red suspension.

Following incubation, the \textit{V. atypica} Se\textsuperscript{II} solutions were filter sterilised under an N\textsubscript{2} atmosphere through 0.22\mu m micropore filters. The biogenic Se\textsuperscript{II} solutions were then split into 15ml aliquots and the pH was adjusted to 11.2 with NaOH.
2.2 **Abiotic synthesis of Se**

Abiotic sodium hydrogen selenide was synthesized by adding sodium borohydride (1.15g, 30.5mM) in 12.5ml of degassed deionised water to grey selenium powder (1.15g, 14.5mM) suspended in 12.5ml degassed deionised water under an N₂ atmosphere, at room temperature and with stirring (17). The resulting, virtually colourless solution of NaHSe was filtered to remove crystals of Na₂B₄O₇·10H₂O that formed upon cooling of the solution after the exothermic reaction. The NaHSe stock solution was diluted to 5mM with 20mM MOPS buffer, and the pH was raised to 11.2 using NaOH under an N₂ atmosphere.

2.3 **Formation and growth of CdSe quantum dots**

A solution of 10mM Cd(ClO₄)₂ and 30mM reduced glutathione (GSH) was prepared so that the final molar ratio upon mixing would be 2:1:3 (Cd:Se:GSH). High purity N₂ was bubbled through the Cd²⁺-GSH solution to remove O₂ from the solution. The pH was then adjusted using NaOH under an N₂ atmosphere to 11.2. Solutions of abiotic and biogenic Se²⁻ and Cd²⁺-GSH were chilled in an ice bath for 1 hour. Aliquots of the Se²⁻ solutions were rapidly injected into equal volumes of the Cd²⁺-GSH solutions under an oxygen-free atmosphere, and were subsequently vigorously shaken for 30 seconds until a yellow precipitate was observed. The GSH-CdSe suspension was then transferred aerobically in to a three-necked round bottom glass and refluxed on a heating mantle at 100°C. Aliquots were taken at 4 minute time intervals until no suspension remained. Aliquots were immediately quenched in liquid nitrogen and stored at -80°C prior to analysis.

2.4 **Characterisation of CdSe quantum dots**

The UV/Vis absorption spectra of the reaction suspension aliquots were measured with an Analytik Jena Specord S600 (Analytik Jena AG, Germany). Photoluminescence spectra were taken with a Gilden Photonics Fluorosens (Guilden Photonics Ltd., UK), using a constant excitation wavelength of 365nm.
HR-TEM images were taken using an FEI Tecnai F20 FEGTEM (200kV), fitted with an Oxford Instruments 80mm$^2$ XMAX SDD EDX detector and a Gatan Orions SC200 CCD. TEM samples were prepared by dropping an aliquot of QDs in water onto a 400-mesh carbon-coated copper grid (Agar Scientific, UK). Particle sizing was via analysis of TEM images, with more than 100 particles assessed at each sample time point for statistical accuracy.

Figure 1. (A) Abiotic and (B) biogenic GSH-CdSe quantum dots fluorescing under 365nm UV irradiation at different reflux time points.
2.5 Protein identification

To determine the presence of protein in the selenide solutions, fractions (25μL) of the abiotic and biogenic Se\textsuperscript{II} solutions were run in an 18% Tris-HCl gel with Tris/glycine/SDS running buffer. Kaleidoscope (BioRad) prestained molecular weight ladders were run on both sides of the gel and gel-separated protein bands were detected using Imperial Protein Stain. Bands of interest were excised under sterile conditions for trypsin digestion. Tryptic peptides were analysed using liquid chromatography-based high-resolution tandem mass spectrometry (LC-MS/MS). All samples were searched first against the SWISSPROT database searching all species, and then against either a species specific search in UNIPROT, or against a species specific database.

2.6 XAS analyses

X-ray absorption spectroscopy (XAS) analyses of the S K-edge were undertaken at the Diamond Light Source, Oxfordshire, UK on the microfocus spectroscopy beamline I18 with the aim of examining the structural relationship between the sulphur component of the glutathione capping agent and the CdSe nanocrystals. A range of standards were assessed, including oxidised and reduced forms of glutathione, elemental sulphur, sodium sulphite and sodium sulphate (figure 7). To remove the impact of unreacted glutathione and remnant MOPS buffer and AQDS, GSH-CdSe was concentrated and purified by resuspension in the nonsolvent 2-propanol as previously described (13, 28).

Further XAS experiments at the Se K-edge (ca. 12654eV) were carried out on the high brilliance X-ray spectroscopy beamline ID26 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France, using a <111> double Si crystal monochromator as described previously (21). The aim was to examine the differences between the formation of chalcogenide QDs from abiotic and biogenic selenide precursors. In this case, the development of ZnSe with the use of β-mercaptoethanol as a capping agent (βME-ZnSe) was used as an analogue system to CdSe. Owing to the speed of the reaction, which exceeds that which can be seen by QUEXAFS, a novel, in situ, time-resolved experiment was carried
out using an injection cell system. The βME-ZnSe was synthesised by injecting equal volumes of an anaerobic solution containing 10mM ZnCl₂ and 24mM β-mercaptoethanol solution and an anaerobic solution of the appropriate Se²⁻ (5mM) to produce final concentrations of Zn, Se and βME of 5, 2.5 and 12mM respectively. Initial XAS scans were taken across the Se K-edge energy after the injection of equal volumes of degassed, deionised water and the abiotic or biogenic Se²⁻ solutions, and also after the injection of the Zn²⁺-βME and the abiotic or biogenic Se²⁻ solutions (figure 8). From these, it was possible to discern two regions of significant difference between the Se²⁻ and ZnSe phases, at 12661eV and 12667eV, which allowed the reaction and development of ZnSe products to be monitored. The sample cell was then flushed with anaerobic, deionised H₂O before re-use. The multi element Ge detector was set to analyse at the specified energy with the maximum refresh rate of 0.5ms during the injection of Zn²⁺-βME and Se²⁻. The sample cell was then flushed and repeated for both stated energies for the abiotic and biogenic Se²⁻ solutions.

3. Results and Discussion

3.1 Particle characterisation and development
The GSH-CdSe suspension was noted to change colour from yellow to red during refluxing, corresponding to the growth of quantum dots. The fluorescence under UV illumination at 365nm wavelength of aliquots removed at 4 minute time intervals is shown in the photograph in figure 1. The UV/vis absorption spectra of these GSH-CdSe aliquots are shown in figure 2. Both sample sets display primary and secondary absorption peaks for the 0 and 4 minute reflux timepoints; at 356nm and 408nm for the abiotic samples, and at 314nm and 334nm for the biogenic samples. The relative intensity of the secondary, lower wavelength absorption peaks decreases with increasing reflux time for both abiotic and biogenic samples. The peak absorption wavelength for both sets of samples increases with increasing reflux time (figure 2), and a second
absorption peak evolves in the abiotic sample sets approximately 30nm above the primary absorption peak.

![Figure 2](image)

Figure 2. UV/Vis (top) and photoluminescence (bottom) spectra for GSH-CdSe QDs synthesised from abiotic Se\(^{II}\) (left) and biogenic Se\(^{II}\) (right). Arrow (bottom left) and inset (bottom right) highlight secondary peaks.

To quantify the photoluminescence observed in figure 1, the GSH-CdSe quantum dots were analysed using a constant excitation wavelength of 365nm and the results are shown in figure 2. The peak emission wavelength for the abiotic GSH-CdSe prior to reflux is at 510nm, which increases to 590nm following 20 minutes refluxing. Comparatively, the biogenic QDs prior to reflux also have a peak emission wavelength of 510nm, which increases to 531nm following refluxing for the same period, 59nm lower than that of the abiotic QDs. A large shift in emission spectra is observed for the abiotic QDs between 4 and 12 minutes refluxing time, which is not observed in the biogenic photoluminescence spectra. Secondary emission peaks are observed forming in the abiotic GSH-CdSe photoluminescence spectra, occurring at 117nm above
the peak emission wavelength (figure 2), becoming more prominent with prolonged reflux time. The biogenic spectra initially show a secondary emission peak occurring 13nm above the primary emission wavelength (figure 2); subsequent refluxing removes this peak.

Figure 3. HRTEM imaging showing (A) Abiotic and (B) biogenic GSH-CdSe, showing QDs synthesised after 8 minutes refluxing at 100°C. Individual crystals are highlighted. (inset) The crystal lattice is visible, and the lattice spacing of 3.5A corresponds to the (002) plane of hexagonal cadmoselite.

HR-TEM was employed to characterise the particle size, shape and morphology of the QD reflux aliquots (figure 3). Spherical, crystalline CdSe particles with diameters of less than 8nm were discernable, and measurements taken of lattice fringes correspond to d-space values of planes within hexagonal CdSe (cadmoselite, ICDD 00-008-0459).

Particle size distributions were calculated using HRTEM images, and the resulting histograms are shown in figure 4. As prepared abiotic and biogenic QDs have equal average particle diameters of 2.4nm (±0.63nm and 0.59nm, respectively). An increase in size distribution is noted for the abiotic QDs after 8 minutes reflux, with an average particle diameter of 3.2nm ±0.78nm compared to 2.9nm ±0.59nm for the biogenic QDs. Following 20 minutes reflux, average
particle diameters have increased to 3.8nm ±0.83nm and 3.6nm ±0.72nm for abiotic and biological QDs, respectively. Figure 5 shows that increasing the temperature of reflux dramatically increases the rate of particle growth for the abiotically synthesised GSH-CdSe QDs, however particle growth for biogenically synthesised QDs is much slower and photoluminescent emissions indicate particle growth rate above 96°C remains constant. Biogenic GSH-CdSe QDs also display a narrower particle size distribution, although PL spectra (figure 2) and particle size distributions from HRTEM (figure 4) for both abiotic and biogenic QDs show a non-Gaussian size distribution with a tail extending to the high particle sizes.

![Figure 4](image.png)

**Figure 4.** Particle size distributions as determined by HRTEM. (A) Abiotic GSH-CdSe particles at (i) 0 minutes, (ii) 8 minutes and (iii) 20 minutes reflux time. (B) Biogenic GSH-CdSe particles at (i) 0 minutes, (ii) 8 minutes and (iii) 20 minutes reflux time. Gaussian distributions for the average particle size are overlaid. For statistical analysis, over 100 particles were used from each time point.

The results of these analyses show that biogenic Se\textsuperscript{II} is a viable alternative to the use of abiotically generated Se\textsuperscript{II} in the formation of metal selenide quantum dots, alleviating the need for highly toxic and strongly reducing compounds.
Analysis of the optical absorption and emission spectra (figure 2) shows that biogenic Se\textsuperscript{II} can be used as a precursor for the synthesis of highly fluorescent nanoparticles comparable to abiotic equivalents. Particle sizing in conjunction with UV/vis and PL emission spectra show that the growth of biogenic QDs occurs at a slower rate than with abiotically generated Se\textsuperscript{II}, suggesting the presence of bacterially derived moieties, such as proteins, inhibit the growth of CdSe QDs.

![Graph showing comparative particle growth during reflux](image)

*Figure 5. Comparative particle growth during reflux as (solid lines) peak emission wavelength, (dashed lines) particle growth derived from photoluminescence peak emission wavelength as a function of reflux temperature and (shapes) HR-TEM determined particle size.*

The reason for the more consistent particle size of the biotic samples could be attributable to a particle size control dictated by coordinating bacterially-derived moieties, such as previously reported for sulphurous peptides (4, 8-10, 25, 36, 41). The development of the large particle size fraction is a deleterious characteristic of aqueous QD synthesis techniques in comparison with organometallic synthesis routes, and although the presence of bacterial proteins
leads to an increased control of particle growth by reducing particle growth rate (figure 5), a post-preparative purification step as used by Gaponik et al (13) may be required.

3.2 Protein identification

The impact of secreted bacterial and fungal proteins on the nucleation and growth of metal chalcogenide quantum dots has been observed previously (2, 3, 7, 8, 10, 36). In order to determine the nature of the bacterially-derived moieties in the biogenic Se\textsuperscript{II} solution in this study, both the biogenic and the abiotic Se\textsuperscript{II} solutions were analysed for protein content by SDS-PAGE (figure 6).

![Figure 6. SDS-PAGE (gel) image of biogenic Se\textsuperscript{II} produced by V. atypica and abiotic Se\textsuperscript{II}. Molecular weight standards are to the right and left. Red staining at the bottom of the gel is due to the precipitation of elemental selenium particles.](image)

No protein bands were visible in the abiotic Se\textsuperscript{II} solution; however the red-orange band at the end of the gel for both the biogenic and abiotic samples indicates the precipitation of elemental selenium nanoparticles as a result of
oxidation of the selenide solution. The biogenic Se$^{II}$ solution contained a range of proteins of varying mass, of which one was putatively identified as the alpha-subunit of methylmalonyl-CoA decarboxylase, similar to that identified in *Veillonella parvula*, and likely originating from *V. atypica*.

3.3 XAS Investigations

Bonding of the reduced glutathione capping agent

The coordination environment of sulphur in the reduced glutathione capping agent associated with the CdSe quantum dots was investigated using XAS at the S K-edge; the XANES results are shown in figure 7, along with relevant model compounds. The XANES spectra of the model compounds show a range of spectral shapes and peak energies with an approximately 10eV difference in peak energy between elemental S and S$^{VI}$.

The spectral profile and absorption edge energy at the S K-edge obtained for the precipitated GSH-CdSe QDs show that S is present in a reduced form, and closely resembles the CdS standard at ca. 2473eV (figure 7). There is no evidence for the presence of S=S bonds as in oxidised GSH, or S-O bonds as the oxidised sulphate or sulphite forms. There are two possible Cd-S coordination environments within the samples tested; (i) the Cd-SR bond, where R is the glutathione molecule, between Cd atoms exposed at the surface of the nanoparticles and the S of the cysteine component within glutathione, in which the Cd-S bonds show similar character to those observed for xanthate attachment to ZnSe (26); and (ii) structural incorporation of free S into a mixed CdSe$_{1-x}$S$_x$ phase, due to the decomposition of glutathione during reflux in alkaline, aqueous media, as reported for the incorporation of S into CdTe quantum dots (13, 30). Gaponik et al (13) postulated that excess thiol, coupled with decreasing free Se$^{2-}$ concentrations as particles grow during reflux, increases the proportion of S structurally incorporated, thereby causing an increasing S:Se ratio in the outer layers of the particle.
Figure 7. S K-edge XANES spectra showing model compounds compared to the experimental GSH-CdSe sample. From the bottom: sodium sulphate, sodium sulphite, reduced and oxidised glutathione, elemental sulphur, the experimentally derived biogenic GSH-CdSe, and CdS.
However, the fact that the size range of the QD’s is limited to below 4nm is evidence that the Cd-SR bond is playing a role in restricting particle growth. Differentiating between sulphur attached to cadmium at the surface and sulphur structurally incorporated into the outer CdSe layers is not possible using standard XANES techniques. Understanding the relationship between sulphurous capping agents and metal selenide QDs, along with determining the relative proportions of the Cd-SR and the CdSe$_{1-x}$S$_x$ during particle growth is challenging and requires collection of an extended x-ray-absorption fine structure (EXAFS) time course data set, at the $S$ K-edge, covering QD formation and growth during reflux, which will be the focus of future research.

Selenide and ZnSe development

XAS was used to analyse and compare the rate of βME-ZnSe nucleation from biogenic and abiotic Se$^{II}$-starting materials, in order to determine the impact of extracellular biological materials on the kinetics of formation of cadmium-free ZnSe QDs (figure 8). As with S, an increase in oxidation state of Se at the $K$-edge is easily discernable by an increase in absorption edge energy (7.5eV between Se$^0$ and Se$^{VI}$).

The results presented here show that the initial biogenic Se$^{II}$-solution was composed predominantly of Se$^{II}$ with a peak at 12653.5eV, but with the possible presence of a minor oxidised component as indicated by the broad bimodal lineshape.

The stability of the abiotic and biogenic Se$^{II}$ solutions was compared by repeated scanning across the Se $K$-edge for 15 minutes (figure 8). Over this time period, the biogenic Se$^{II}$-solution remains as a largely reduced form indicating that it is stable and does not undergo oxidation. However, the abiotic Se$^{II}$-peak shifts dramatically towards a principally Se$^{VI}$ form, indicating that the abiotic Se$^{II}$ is susceptible to oxidation, possible induced by the X-ray beam. It is possible that the proteins identified in the biogenic Se$^{II}$-solution may coordinate with the selenide and act as stabilising agents, limiting oxidation.
Figure 8. Se K-edge XANES spectra for model compounds (top) iron selenide, red elemental selenium, black elemental selenium, selenomethionine, sodium selenite, sodium selenate and experimental products (bottom) investigating the stability of the biogenic and abiotic Se^{II} solutions before and after exposure to the synchrotron x-ray beam for 15 minutes.
Figure 9. Se K-edge x-ray absorption spectra for the biogenic (top) and abiotic (bottom) Se$^{ll}$ solutions (black lines) and β-mercaptoethanol capped ZnSe (grey lines) formed following addition of ZnCl$_2$. The energies used for the time resolved scans are indicated.

The addition of ZnCl$_2$-βME to both biogenic and abiotic Se$^{ll}$ stocks leads to the rapid formation of βME-ZnSe nanocrystals, as shown in figure 9. From these spectra, there is a discernable difference in absorption intensity at 12661eV and 12667eV between the Se$^{ll}$ solutions and βME-ZnSe suspensions for both the biogenic and abiotic samples. The change in intensity at these energies defines the rate of nucleation of ZnSe QDs, both in the presence and absence of extracellular biological material (figure 10). First order derivatives of the
obtained time-resolved spectra highlight differences in the rate of transformation from the Se$^{II}$ phase to the ZnSe phase, with the biogenic Se$^{II}$-samples displaying a wider peak than abiotic counterparts; full-width half-maximum values for these reactions are 1.25ms and 1.23ms for the biogenic Se$^{II}$-samples at 12661eV and 12667eV, respectively and 0.77ms and 0.74ms for the abiotic Se$^{II}$-samples at 12661eV and 12667eV.

Figure 10. Time resolved energy scans (main image: 1$^{st}$ order derivates, inset: time-resolved scans) for (A) biogenic ZnSe at 12661eV, (B) biogenic ZnSe at 12667eV, (C) abiotic ZnSe at 12661eV and (D) abiotic ZnSe at 12667eV.

It is possible that proteins present in the biogenic Se$^{II}$ are also implicated in decreasing the rate of reaction between Zn$^{II+}$ and Se$^{II}$, as shown in figure 10 where the FWHM for two independent biogenic Se$^{II}$ reactions are twice that for those obtained with abiotic Se$^{II}$. However, there are limitations associated with these results, as the FWHM reaction times calculated are of the same magnitude as the maximum detector refresh rate (0.5ms), although the repetition of the results from two separate biogenic Se$^{II}$ reactions and two
abiotic Se\textsuperscript{II} reactions suggests these findings are valid; further investigations using ultrafast time-resolved detectors is warranted.

4. Conclusions

The technological, industrial and biological advances using semiconducting, nanoparticulate ‘quantum dots’ warrants the investigation of techniques aimed at reducing the economic and environmental costs associated with their manufacture. This study has compared the formation of metal selenide quantum dots from bacterially derived Se\textsuperscript{II} with quantum dots synthesised from traditional chemical precursors.

It has been shown that the use of bacterially derived Se\textsuperscript{II} in the manufacture of metal selenide quantum dots increases control on the rate of formation and growth of the QDs, with indications of a decrease in initial reaction rate and a narrower size distribution of particles in comparison to abiotic counterparts, even in the presence of thiol capping agents. The decreased particle size distribution and the reduced particle growth rate allow for tighter size constraints with biotic materials compared to wet synthesis experiments with abiotic Se\textsuperscript{II}. This may help to alleviate one of the disadvantages of wet synthesis methods over organosynthetic approaches.

Protein gel electrophoresis has shown that bacterial contributions to the synthesis of technologically relevant materials is not limited to direct formation of the Se\textsuperscript{II} solutions; extracellular biological materials such as the proteins indentified appear to play an important role in Se\textsuperscript{II} stability after the cells have been removed. The mechanism of GSH attachment to the QD is identified. Novel, in situ time-resolved XAS experiments have also demonstrated that the presence of bacterially derived proteins provides increased control over QD size by reducing the rate of initial particle formation.

This research represents an alternative, ‘green’ pathway that utilises the advantages of biosynthetic techniques, namely the cheap, low temperature and relatively safe synthesis of Se\textsuperscript{II} without the use of highly toxic and expensive precursors. The results also highlight the stability of the precursors and
enhanced control of QD synthesis that can be obtained in the presence of biological coatings.

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Chapter 9

Conclusions and future work
1. Conclusions

The biogenic synthesis of nanomaterials has been demonstrated repeatedly over the last 20 years, with several species of yeast and numerous, disparate bacterial species known to produce semiconductor nanomaterials which display quantum confinement effects comparable to abiotically synthesised counterparts. The initial research hypothesis pursued here was that areas containing elevated concentrations of particular elements (in this case Se) would contain a microbial community well adapted to these high concentrations, displaying resistance, detoxification and possibly respiratory mechanisms that could be adapted for use in the bacterial synthesis of technologically, industrially or chemically relevant nanomaterials.

The first step in this process was the identification of regions that may contain bacterial species capable of being utilised in selenium nanoparticle synthesis (chapter 4). The seleniferous field site in Co. Meath, Ireland, was identified by soil elemental maps (5) and published reports of Se toxicity in animals (2, 3, 13) and soil Se enrichment (6, 12). Soil cores taken from this site contained a horizon with a Se content of 156ppm, and elevated Se concentrations were noted throughout the soil profile. The Se was strongly correlated with total organic carbon, which GC analysis indicates is primarily higher plant material in origin. XAS analysis showed that Se is found as a reduced organic phase similar to selenomethionine, and is recalcitrant to bacterial degradation under microcosm conditions as tested. These findings are in agreement with Parle and Fleming (12), who theorize that a phytococoncentration mechanism is responsible for retaining and concentrating incoming oxidised Se from drainage of the nearby seleniferous shale components of the Lucan Formation.

Molecular ecological analysis of the bacterial community found within the seleniferous horizon showed that drastic alterations in community structure occur following incubation with 5mM Se$^{VI}$, which became rapidly reduced to the less toxic, immobile Se$^{0}$. These large changes infer that microbial resistance to high levels of Se was not widespread throughout the community, despite the naturally high Se content. These findings show that the targeted search for bacterial species in the
environment is complicated not only by the search for areas which contain elevated levels of the element in interest, but also by the bioavailability, geochemical state of the element and specific interactions with the microbial community.

Complimentary to the investigation of a natural environment was the investigation of the interactions of the well characterised, model environmental isolate *Geobacter sulfurreducens* with the chalcogens Se and Te. This was to test the hypothesis that environmentally isolated species are suitable for bionanomaterial production (chapter 5). It has been demonstrated that *G. sulfurreducens* is incapable of growth in the presence of either of these oxyanions at concentrations exceeding 10μM, findings which are unexpected given the large concentration of c-type cytochromes expressed; c-type cytochromes have been shown capable of SeVI reduction in isolation (1). These findings suggest the absence of an effective detoxification mechanism. Despite this inability to grow with or in the presence of Se or Te, ‘resting cell’ cultures of *G. sulfurreducens* are capable of producing significant quantities of Se0 precipitates, and it is possible to produce Se0 nanospheres of a predetermined size and size distribution and which display an increased stability in comparison to chemically synthesised counterparts. This ability to ‘tune’ the nanoparticles produced greatly increases the viability of bionanomineralisation as a technique to produce relevant nanomaterials.

The application of these as-prepared biogenic Se0 nanospheres in a real-world environment was explored with the application to sequestration of volatile Hg species (chapter 6). Se0 has been previously demonstrated to be well suited to the capture of volatile Hg0, with the formation of a stable, immobile HgSe phase (7, 10). This research directly compared the Hg0 retention ability of biogenically and abiotically prepared Se0 nanospheres in laboratory- and *in situ*-based experiments, finding that despite a lower initial rate of Hg0 capture, an increased long term stability displayed by the biogenic Se0 was desirable for long term, slow Hg0 release applications. Chapters 5 and 6 together describe the formation and application of Se0 nanoparticles synthesised by the model environmental isolate *G. sulfurreducens*, demonstrating that this biochemically versatile bacterium is capable of producing
useful bionanomaterials despite having low tolerances to either of the chalcogens tested.

The bacterial biogenic synthesis of technologically relevant metal selenide nanomaterials was the focus for the remaining research of this project. Direct comparisons were drawn between the environmentally isolated bacteria G. sulfurreducens and Shewanella oneidensis and the clinical isolate Veillonella atypica (chapter 7). The ability to reduce supplied Se oxyanions to the Se^{II} phase is highly desirable, as this circumvents the necessity for the chemical generation of Se^{II}, which requires the use of a number of highly toxic and expensive chemicals under hazardous conditions. The clinical isolate V. atypica was noted to be extremely well suited to the rapid generation of large quantities of Se^{II} in the presence of the electron shuttling compound anthraquinone-2,6-disulphonate (AQDS), and is thought to use a biochemical pathway that is unlike the cytochrome-mediated reduction noted for G. sulfurreducens and S. oneidensis.

Accordingly, biogenically prepared Se^{II} solutions by V. atypica were used in the production of semi-conducting metal selenide ‘quantum dots’ (chapter 8). The development of alternative, economically viable, ‘green’ synthesis routes are highly desirable as quantum dots are increasingly developed for technological and biological applications. Biogenic quantum dots are shown to be comparable to chemically synthesised counterparts. Furthermore, biological components produced alongside Se reduction have been shown to influence the subsequent crystallisation and growth of metal selenide phases, acting to decrease growth rate (thereby increasing control on particle size) and increase particle stability.

The use of bacteria to produce bionanomaterials represents a novel technique to reduce economic and environmental costs associated with the production of next-generation technologies. The search for bacteria suited to the formation of useful Se nanomaterials within the environment is greatly complicated due to the numerous forms Se can be found; the solubility, bioavailability and toxicity of Se are dependent on oxidation state, in turn typically governed by geochemical conditions. The search for natural microbial consortia or isolates will need to move to more extreme,
bioavailable concentrations of the element under investigation, and possibly more extreme environments where these bacteria thrive.

Despite this, this research has shown that it is possible to successfully utilise environmentally isolated bacteria in the production of functional chalcogen and metal chalcogenide nanomaterials, and has shown that biogenic nanomaterials display distinct advantages when compared against chemically synthesised equivalents, such as increased stability or increased control over the rate of particle formation. In conclusion, the bacterial production of chalcogen and chalcogenide nanomaterials represents a promising alternative to chemical synthetic methods, offering the distinct possibilities of economic and environmental advantages whilst also capable of producing novel bionanomaterials.

2. Future work

This research has revealed potential pathways to the biotechnological development of nanoparticles and as well as factors relating to the geochemical behaviour of Se in the environment. Also revealed are future research directions to fully understand the search for, and utilisation of, environmentally isolated bacteria with the aim of generating novel nanomaterials and nanomaterial synthesis routes.

These future work areas have been broken down into the specific areas that they address:

i. The search for suitable bacteria in the environment

Although nearly 400 times the global average concentration of Se in soils, the Se contained within the seleniferous horizon at the Co. Meath field site was largely unavailable to soil microorganisms. As such, further areas of natural or anthropogenic enrichment of Se need be sought. At these sites a more detailed analysis of the Se speciation using synchrotron radiation (high resolution EXAFS) will
facilitate interpretations. This coupled with community analysis will, at the very least, provide a deeper insight into environmental responses to Se. A complicating factor in these extreme environments is the accumulation other toxic metals (for instance high U and Cd in Co. Meath), although this represents an opportunity to examine the environmental behaviour of these elements and the microbial responses to them.

ii. Investigating the toxicity of Se and Te in G. sulfurreducens

The low resistance to Se and Te oxyanions displayed by G. sulfurreducens was a surprising result as cytochrome-mediated reduction of Se and Te was implicated for resting cell cultures of both G. sulfurreducens and S. oneidensis. Published literature suggested that S. oneidensis is capable of growth in Se concentrations exceeding 2mM (9), whereas the findings here showed G. sulfurreducens would not grow in Se or Te concentrations exceeding 10μM (chapter 5). This discrepancy may be due to the absence of glutathione reductase in the G. sulfurreducens genome. Further investigations in this area could increase knowledge of the bacterial biochemical reactions of Se and Te, and attempts to increase the resistance of G. sulfurreducens to Se and Te oxyanions may allow for the continual, rather than batch, production of chalcogen nanomaterials. Further investigations into Te oxyanion resistance mechanisms may also allow for the biogenic synthesis of Te\(_{\text{II}}\) solutions, which would allow for the biosynthesis of the technologically important metal telluride nanomaterials.

iii. The evolution of Hg\(_{\text{0}}\)\(_v\) from contaminated museum specimens

Investigations into the application of Se\(_{\text{0}}\) to Hg\(_{\text{0}}\)\(_v\) capture found that the bacterial Se\(_{\text{0}}\) nanomaterials were well suited to the sequestration of Hg\(_{\text{0}}\)\(_v\) over extended periods, owing to the increased particle stability displayed. It is likely that bacterially secreted polymeric substances are acting to passivate the surface of Se\(_{\text{0}}\) particles, as previously seen (4, 7, 8, 11, 14), and it is suggested that future work should investigate the relationship between bacterially secreted proteins and Se\(_{\text{0}}\).
nanoparticles. The mechanism of Hg\textsuperscript{0} evolution from the herbarium specimens is still unclear. Detailed studies of the speciation of Hg on specimens (XRF/XANES mapping) and organometallic Hg analysis would provide an insight into the transformation from original HgCl\textsubscript{2} phase used.

iv. The biogenic synthesis of metal selenide quantum dots

This research has found that biogenic Se\textsuperscript{II} solutions are comparable to chemically synthesised Se\textsuperscript{II} for the formation of metal selenide quantum dots, and the presence of bacterial secreted polymeric substances in the precursor Se\textsuperscript{II} solution alters the rate of particle formation, inferring increased control over particle size and distribution. Preliminary identification of proteins found within Se\textsuperscript{II} solutions was undertaken in this work, however further research could build upon this by isolating and purifying bacterially secreted proteins, and determining the effects these have upon quantum dot formation. This falls into the very important area of capping agents for QDs, a major issue in maintaining monodisperse particles. Ultimately, it may be possible to enhance bacterial secretion of specific proteins to replace the requirement of externally supplied organic capping agents. Finally, the biogenic Se\textsuperscript{II} solutions were principally used to synthesise the well characterised, heavy-metal bearing CdSe and ZnSe phases. Owing to international restrictions in the use of heavy metals, further research could be undertaken in exploring the role biogenic Se\textsuperscript{II} solutions could play in the formation of novel, heavy metal free quantum dots.

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