

PROTEIN EXTRACTION FROM SEDIMENT BOUND MICROBES
CAPABLE OF BIOREMEDIATION FOR PROTEOMIC STUDIES

By

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Abstract

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Sediments contaminated with heavy metals pose a significant risk to human health and the environment. Remediating these contaminated sediments has historically been difficult due to the cost and labor involved. Bioremediation offers an approach for cleaning up pollutants by enhancing biodegradation processes that occur naturally. For example, certain sediment microbes have the capacity to reduce heavy metals into an insoluble form, which prevents these metals from leaching into waterways. Characterization of geomicrobial protein expression (proteomics) provides information needed to better understand the unique biological pathways that make these remediation processes possible. More importantly, more thorough knowledge will permit better nutrient enrichment of contaminated sites, increasing microbial production of factors important in heavy metal reduction. A significant challenge in studying sediment microbe protein is their initial dissociation from the sediment particles. Removal of intact bacterial cells, prior to lysis, has proven to be ineffective. This limits characterization of the complete representation of the microbial community. More problematic, direct *in-situ* lysis of bacterial cells leads to extreme protein adsorption due to the heterogeneous nature of sediment and the physiochemical properties of protein. Therefore, blocking protein adsorption sites on sediment followed by lysis of bacterial cells *in-situ* is

investigated in this project. Aspects of proteins: isoelectric point (pI), mass and hydrophobicity as well as sediment properties, such as surface charge, sediment particle variation and pore space heterogeneity all play major roles in achieving effective protein removal from sediment for proteomic analysis. Various methods for extracting protein from sediment with emphasis on methods that are compatible with tryptic digestion and mass spectrometric analysis are tested by the addition of lysed *Escherichia coli* protein to representative sediment samples. These methods include testing a priori treatment of sediment samples with amino acids to block binding sites, testing the best binding condition to enhance binding agent efficiency and the development of a protein desorption buffer. The results conclude that it is possible to significantly increase protein identification through blockage of binding sites on sediment combined with use of a desorption buffer.

TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	iii
ABSTRACT	iv
LIST OF TABLES	xi
LIST OF FIGURES	xii
CHAPTER	
1. BIOREMEDIATION	
1.1 Introduction and background	1
1.2 Principles of bioremediation	2
1.3 Dangers of uranium	3
1.4 Current bioremediation research	4
1.5 Overview of proteomics	5
1.5.1 Properties of protein	6
1.5.2 Proteomic sample preparation	8
1.5.2.1 Cell disruption	8
1.5.2.2 Chaotropic agents	10
1.5.2.3 Reducing agents	11
1.5.2.4 Surfactants	11
1.5.2.5 Protein Digestion	11
1.5.3 Proteomic mass spectrometry	12
1.5.4 Proteomic data analysis	13

1.6 Importance of the proteomics of bioremediation capable microbes	15
1.6.1 Metaproteomics	15
2. EXTRACTON OF SEDIMENT MICROBIAL PROTEIN	
2.1 Introduction	17
2.2 Great plate count anomaly	17
2.3 Biofilms	18
2.4 Properties of sediment	20
2.4.1 Particle size	20
2.4.2 Composition	21
2.4.3 Charge	23
2.5 Extraction techniques	24
2.5.1 Analysis of in-direct extraction	24
2.5.1.1 Microorganism adsorption to sediment surfaces	24
2.5.1.2 Pore spaces	26
2.5.1.3 Analysis of previous research	27
2.5.1.4 Discussion	29
2.5.2 Analysis of the direct extraction technique	30
2.5.2.1 Protein adsorption to sediment surfaces	30
2.5.2.2 Hydrophobic/hydrophilic interactions	31
2.5.2.3 Structural rearrangements	32

2.5.2.4 van der Waals interactions	32
2.5.2.5 Electrostatic interactions	33
2.5.2.6 Sediment mineral composition	34
2.5.2.7 Discussion	34
2.6 Direct extraction with prior blocking of protein adsorption sites	35
2.6.1 Modification of the sediment to produce a non- adsorptive surface	35
2.6.2 Development of a protein desorption buffer	37
2.7 Discussion	41
3. RESEARCH PARAMETERS	
3.1 Introduction	44
3.2 Hydrometer test	44
3.2.1 Hydrometer test results	45
3.3 Mineralogy	45
3.3.1 Mineralogy results	47
3.4 <i>Escherichia coli</i> for protein binding tests	48
3.4.1 <i>E. coli</i> Growth Conditions	48
3.4.2 Tryptic digestion of <i>E. coli</i> samples	48
3.5 Experiments	49
3.6 Mass spectrometer capillary LC-MS analysis	50
3.7 Experimental data analysis	51

4. METHODS AND RESULTS FROM TEST 1 – Binding Sediment	
Adsorption Sites with Amino Acids	
4.1 Amino acid classes	53
4.2 Amino acid binding test procedure	54
4.3 <i>E. coli</i> protein binding	56
4.4 Results	56
4.5 Discussion	61
5. METHODS AND RESULTS FROM TEST 2 – Optimization of	
Binding Conditions	
5.1 Test 2 parameters	63
5.1.1 Control tests	64
5.1.2 pH tests	64
5.1.3 Temperature tests	64
5.1.4 Mechanical dispersion tests	64
5.1.5 Time tests	65
5.1.6 Concentration tests	65
5.1.7 <i>E. coli</i> only control tests	65
5.2 Results	67
5.3 Discussion	70
6. METHODS AND RESULTS FROM TEST 3 – Desorption Buffer	
Test	
6.1 Test 3 parameters	76
6.2 Results	77

6.3 Discussion	79
6.4 Final conclusions	83
6.5 Future directions	84
REFERENCES	85

List of Tables

Chapter	3	Research Parameters	
Table	3.1	Mineralogy of the Old Rifle Background Sediment	47
Chapter	4	Methods and Results from Test 2	
Table	4.1	Standard amino acids and side chain properties at pH 7.0	51
	4.2	Increasing hydropathy index of amino acids from hydrophilic to hydrophobic	52
	4.3	Unique combinational peptides and proteins identified in Test 1	57
	4.4	Range between biological replicates for each treatment in Test 1	60
Chapter	5	Methods and Results from Test 2	
Table	5.1	Unique combinational protein and peptides identified in Test 2	67
Chapter	6	Methods and Results from Test 3	
Table	6.1	Unique combinational protein and peptides identified in Test 3	77
	6.2	Protein functional categories identified per treatment in Test 3	82

List of Figures

Chapter	1	Bioremediation	
Figure	1.1	The condensation of two amino acids to form a peptide bond	7
	1.2	Representation of PCT-mediated protein extraction	10
Chapter	2	Extraction of Sediment Microbial Protein	
Figure	2.1	Size fractionation of soil constituents	21
	2.2	Representation of sediment microbe habitat	26
	2.3	Nycodenz density gradient scheme	29
Chapter	3	Research Parameters	
Figure	3.1	U.S. Textural Triangle (USDA) Classification of Old Rifle, CO background sediment	45
	3.2	Flowchart of Test 1 - Amino acid binding experiment	53
Chapter	4	Methods and Results from Test 1	
Figure	4.1	Flowchart of Test 1 - Amino acid binding experiment	53
	4.2	Unique combinational protein and peptides identified in Test 1	58
	4.3	Reproducibility of protein and peptide identifications from Test 1	59
	4.4	Percentage of protein functional categories adsorbing to 10% PPAA treated sediment compared to protein	

		functional categories adsorbing to untreated sediment	61
Chapter	5	Methods and Results from Test 2	
Figure	5.1	Flowchart of Test 2 - Binding conditions experiment	66
	5.2	Unique combinational protein and peptides identified in Test 2	68
	5.3	Reproducibility of protein and peptide identifications from Test 2	69
	5.4	Protein loss by functional category due to sample manipulation and digestion	70
	5.5	Bimodal pI distribution of proteins identified in Test 2	74
	5.6	Hydrophobicity distribution of <i>E. coli</i> proteins identified in Test 2	74
	5.7	Mass distribution of <i>E. coli</i> proteins identified in Test 2	75
Chapter	6	Methods and Results from Test 3	
Figure	6.1	Unique combinational protein and peptides identified in Test 3	77
	6.2	Reproducibility of protein and peptide identifications from Test 3	78
	6.3	Bimodal pI distribution of combinational <i>E. coli</i> proteins identified in Test 3	80
	6.4	Hydrophobicity distribution of combinational <i>E. coli</i> proteins identified in Test 3	80
	6.5	Mass distribution of <i>E. coli</i> proteins identified in Test 3	81

Dedication

To Dani

You have taught me more than any book

Chapter 1

Bioremediation

1.1 Introduction and background

Microorganisms within the environment are constantly breaking down organic matter to release mineral nutrients into a form that is available for primary producers. For a large part of the geological past of the Earth, microorganisms have had primary roles in shaping the natural environment from the degradation of waste products to nutrient cycles. On a grand scale, microorganisms are the backbone of ecosystems and ensure homeostasis of the planet through important enzymatic reactions (Rodriguez-Valera 2004). Microbial enzymes are proteins which can be studied in different natural ecosystems through the emerging field of metaproteomics which is the proteomic analysis of mixed microbial communities (Wilmes and Bond 2006). The dissimilative reactions that bacteria perform to gain energy and to decompose organic matter include aerobic respiration (oxygen reduction), nitrate reduction, sulfate reduction and metal reduction (Lynch and Hobbie 1988). However, should the environment become contaminated with a pollutant such as a heavy metal, some microorganisms will die while others are capable of survival, changing the population. Bacteria which survive are often capable of continuing these reductive reactions thereby degrading or altering the pollutant. Heavy metals pose a significant challenge to remediation because they cannot be destroyed. However, certain dissimilatory metal-reducing microorganisms are capable of reductively precipitating radioactive metals, such as uranium, in contaminated subsurface environments. Precipitation of heavy metals shifts toxic metals from the aqueous phase to a solid phase, decreasing the risk to organisms by lowering metal

bioavailability, toxicity and mobility into waterways (Anderson, Vrionis et al. 2003). Proteomic analysis of these environmentally important microbes includes the direct qualitative and quantitative assessment of the full complement of proteins present in the microbial community through detection with a mass spectrometer. Since bioremediation-capable microbes exist within a complex, heterogeneous environment (sediment and soil), it is essential to develop a robust, effective, protocol to extract the true representation of proteins expressed by the community of microbes present in these sites. Removal of intact bacterial cells has historically been proven difficult due to microorganism/sediment interactions (Lindahl and Bakken 1995). Lysis of the bacteria on the sediment followed by protein extraction is also difficult due to intense sediment-protein interaction. To avoid protein loss, blocking protein adsorption/binding sites on sediment prior to lysis should increase protein recovery for mass spectrometry analysis. Techniques for adsorption site binding with subsequent protein re-solubilization are needed to be adequately characterized.

1.2 Principles of bioremediation

Bioremediation is the use of biological organisms to solve environmental problems by returning contaminated sites to their natural conditions. In the case of land contaminated with heavy metals, bioremediation works by providing metal-reducing organisms with conditions that encourage their rapid growth and thus enabling microbial reduction of the pollutant at a proportionately faster rate than naturally occurring processes. This is accomplished by enhancing the same processes that occur in nature and is therefore safer and less expensive than alternative solutions such as incineration or landfilling of the contaminated materials. It also has the advantage of treating the

contamination *in-situ* so that large quantities of sediment or water do not have to be removed for treatment (Keith-Roach and Livens 2002).

One of the most challenging environmental problems is the contamination of groundwater with radionuclides and metals from former nuclear weapons and nuclear energy sites. Uranium mill tailings are a significant environmental threat due to human health and the ecological risk associated with U-contaminated groundwater plumes.

1.3 Dangers of uranium

Uranium is a naturally occurring heavy metal found as uranium ore and can be extracted for multiple purposes including nuclear energy and weaponry. Uranium mill tailings are the residual waste from the process of uranium extraction from uranium ore. By the end of World War II, uranium was in high demand and hundreds of uranium mines had been activated. The Cold War and the Manhattan Project catapulted the demand even further and left behind a legacy of the environmental management of “contaminated land located at 144 sites in 31 states and a U.S. territory. The total volume of soil, groundwater, and sediment present in highly diverse environments and contaminated with complex mixtures of radionuclides, metals, and organic contaminants may exceed 1800 million cubic meters” (DOE 2007). Uranium mills produced millions of tons of uranium tailings, liquid waste residue, radioactive materials, other [toxic] metals and inorganic contaminants. Due to the urgency during wartime, little or no care was taken to isolate the tailing materials from the environment despite the chemical toxicity and radiological hazards to human health. People can be exposed to the tailings via ingestion, inhalation or direct contact at contaminated sites. Additionally, oxidized uranium is soluble in groundwater is mobile within the subsurface. Uranium has both

chemical and radiological toxicity and is a suspected carcinogen, teratogen and mutagen and a known nephrotoxicant (WHO 2003). Kidney dysfunction is the main chemically induced effect of uranium exposure and although uranium which has not been enriched is weakly radioactive, it does have the potential to cause lung cancer if inhaled (Nordberg 2007). Despite recent clean-up efforts to remediate contaminated uranium mill sites, uranium still remains underground, solubilizing and leaching into groundwater systems. Eventually these networks of groundwater systems lead to rivers and lakes. Microbial bioremediation is being considered as a means to clean up uranium contaminated sites with many studies focused exploitation of natural microbial processes for uranium bioremediation and development of methods to accelerate these processes (Lovley, Phillips et al. 1991), (Lovley, Holmes et al. 2004), (Vrionis, Anderson et al. 2005).

1.4 Current bioremediation research

Current bioremediation research activities include the Department of Energy's (DOE) Integrated Field Research Challenge (IFRC) site which involves the cleanup of an inactive uranium processing mill located in Rifle, Colorado. The original mission of the Rifle mill site was to provide uranium for the United States government. The source of contamination was the tailings that remained after the milling process extracted the uranium. The Old Rifle mill operated from 1924 to 1932 for the recovery of vanadium. Production was altered to include recovery of uranium from 1947 to 1958 (EIA 2005). The land is now part of the DOE's Uranium Mill Tailing Remedial Action program (UMTRA) focused on the *in-situ* immobilization of uranium via the acceleration of microbially mediated reduction of soluble U(VI) (the mobile valence state of uranium) to insoluble U(IV) (uraninite) by adding an electron donor such as acetate (Langmuir 1978).

Biostimulation allows bacteria to use uranium as a terminal electron acceptor during respiration. Microbial respiration requires the reduction of the terminal electron acceptor. In the case of soluble metals, an immobile precipitate is formed removing the contaminant from groundwater and preventing pollutant migration to streams and drinking water (Vrionis, Anderson et al. 2005). Bacteria that utilize metals as terminal electron acceptors are anaerobes which belong to three major biochemical groups: denitrifying, sulfate, and iron reducing bacteria (Lovley, Phillips et al. 1991). The Old Rifle site has experienced significant loss of soluble uranium in connection with a substantial shift in the subsurface microbial community towards members of the *Geobacter* family (Lovley, Phillips et al. 1991). Electron transfer pathways within these bacteria are poorly characterized; therefore efforts to increase the efficiency of *in-situ* bioremediation have been delayed. More complete classification of metal reductases is essential for understanding these mechanisms and application of bioremediation at DOE sites (Lovley, Holmes et al. 2004). As a long-term goal, the DOE seeks to understand processes controlling contaminant mobility in the subsurface and determine whether bacterial iron oxidizers play significant roles in the oxidation of U minerals under environmentally relevant conditions. The DOE would also like to “identify the structure and functional physical mechanism of key proteins involved in direct electron transfer and to elucidate the molecular mechanisms involved in microbial metal reduction” (Bolton 2008).

1.5 Overview of proteomics

Whereas genomics is the study of genes, proteomics is the study of the expressed protein. Proteomics holds additional complexity due to the myriad levels of regulation of

expression as well as the post-translational modifications that can be exhibited by a single protein. As a result, a single genome can display many proteomes dependent on (for instance) responses to disease, cell cycle stages, and environmental conditions including temperature, stress and nutrient availability. A proteome is dynamic, endlessly changing in response to stimuli. Since proteins are the mainstay to cellular function, constant interaction is taking place via biological networks and pathways (Hood 2003). Characterizing the entire protein complement of a cell under a given set of conditions is useful in the study of biological systems by allowing us to understand organism phenotype (Lipton, Pasa-Tolic et al. 2002). Mass spectrometry driven proteomic studies can be divided into a series of steps: digestion of proteins into peptides, separation of the peptides and identification using mass spectrometry (by accurately measuring the masses of peptides generated from the digestion of the protein) followed by the use of software that scan the different protein databases available to identify the protein.

1.5.1 Properties of protein

Proteins are biological macromolecules made up of 20 standard amino acids and they play an essential role in cellular processes, including cell signaling, cell cycle and immune response (Manton 1993). The general structure of the component amino acids includes an α -carbon, a carbonyl group, an amino group, and a side chain. The side chains give the amino acids a variety of different properties including a range of polarities (a hydrophobic to hydrophilic character) and charge. The 20 amino acids can combine and generate an infinite number of possible proteins through peptide bonds via condensation reactions which requires the elimination of a molecule of water formed by a hydrogen coming from the amino terminal of an amino acid and a hydroxyl coming from

the carboxyl group of another amino acid. Elimination of water allows for the formation of the peptide bond (Whitford 2005).

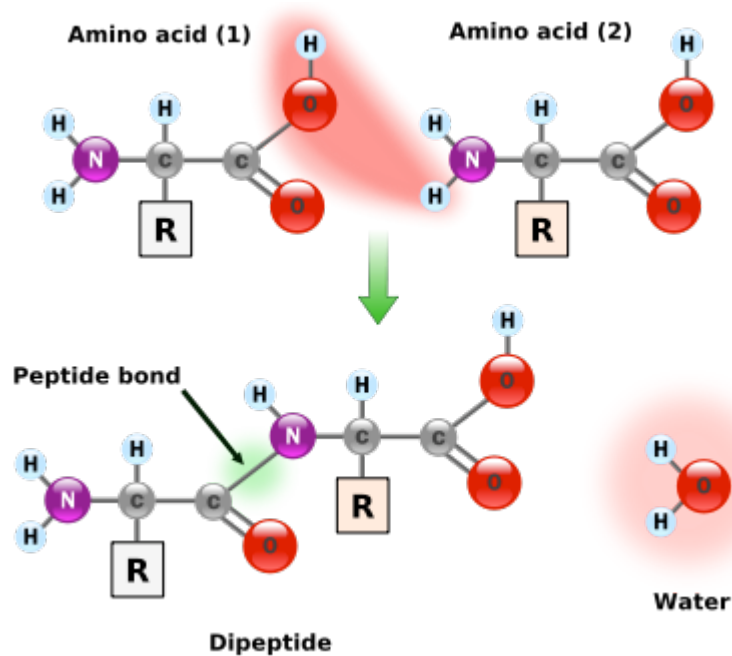


Figure 1.1 - The condensation of two amino acids to form a peptide bond (Mrabet 2007)

Based on the side chain properties, amino acids of a protein can form non-covalent interactions through electrostatic, hydrogen bonding, and hydrophobic affinities. The sulfur group present in cysteines can form disulfide bridges. The oxygen of the carbonyl group can form a hydrogen bond with the hydrogen of the amino group as well (Whitford 2005). These interactions result in the folding of proteins that give them a three dimensional structure. Proteins vary primarily in three different ways including, but not limited to:

- 1) Size - measured as molecular weight, is dependent upon the number and type of component amino acids. The average molecular weight for a protein is 50kD .

- 2) Charge - every protein has numerous charges. The amino terminus is positively charged and the carboxy terminus is negatively charged. Amino acid side chains (R groups) may carry a charge depending on their individual structures. The net charge on a protein is the sum of all the individual charges and varies with pH. At the isoelectric point (pI), the protein has no net charge or the number of positive charges is equal to the number of negative charges. At low pH, proteins (and individual amino acids) become more positively charged (due to gain of a proton). At high pH, they become more negatively charged (due to a loss of a proton).
- 3) Hydrophobicity - the number, type and distribution of nonpolar amino acids within a protein determines its overall hydrophobicity and therefore solubility (Lesk 2004).

1.5.2 Proteomic sample preparation

In order to study a proteome, proteins must be separated and subsequently identified from a biological sample. The first step in proteomic study is sample preparation. Proteins must first be extracted by disruption of the cell membrane followed by solubilization. The proteins must then be reduced, denatured and enzymatically digested into peptides for injection in a mass spectrometer.

1.5.2.1 Cell disruption

In animal cells, a plasma membrane separates the cell contents from the environment, but in some bacteria, fungi and plants the plasma membrane is surrounded by a rigid cell wall. Animal cells are relatively easier to lyse than most microbial cells. Microbes can withstand much harsher conditions (von Hagen 2008). An instrument

commonly used for cell lysis is the Barocycler NEP2320 (Pressure Biosciences, South Easton, MA). The Barocycler utilizes alternating levels of hydrostatic pressure to break apart cells by destabilizing intermolecular interactions. The samples are placed in a specialized tubes equipped with a lysis disk called PULSE (Pressure Used to Lyse Samples for Extraction) tubes. This instrument can generate cycles of pressure from ambient to 35kpsi with nearly instantaneous depressurization time and dwell times of 5 to 60 seconds at any pressure in between (Tao 2007). Pressurization and depressurization forces the sample through the lysis disk providing a shearing action while high hydrostatic pressure acts preferentially on the compressible constituents of the sample. Lipids are the most compressible sample components and dissociate upon depressurization because energy distribution results in destabilization of molecular interactions in the lipid bilayers and other cellular components.

1.5.2.3 Reducing agents.

For complete protein unfolding it is necessary to reduce the intra- and intermolecular disulfide bonds within and between protein subunits. Dithiothreitol (DTT) is a reductant that can be used for these purposes (von Hagen 2008).

1.5.2.4 Surfactants

In order to avoid loss of protein due to aggregation and precipitation it's important to prevent hydrophobic interactions between the hydrophobic protein domains within and between proteins. This is achieved through the use of surfactants or surface active agents. Surfactants have both a hydrophobic tail and a hydrophilic head. The character of the polar head group gives rise to a number of surfactant classifications from ionic (anionic or cationic), non-ionic or zwitterionic (both positively and negatively charged groups but with a net charge of zero) (Walker 2005).

1.5.2.5 Protein digestion

Instead of simply analyzing proteins on the mass spectrometer, they are digested into peptides (or “bottom-up” proteomics) since they are smaller and mass spectrometers are more efficient at obtaining sequence data from them (protein can also be analyzed on the mass spectrometer with a higher level of complexity; this is “top-down” proteomics). Peptides also negate the physiochemical properties of proteins that can be difficult to handle such as solubility and tendency to aggregate. Also, even a small set of tryptic peptides from a specific protein are enough for identification (Sensen 2005).

The most commonly used protease for proteomic analysis is trypsin due to the fact that it very specifically cleaves peptide bonds that are C-terminal to the basic amino acid residues arginine and lysine. This creates peptides in the preferred mass range for

sequencing and results in spectra that are information-rich and easily interpretable. Trypsin generates peptides with an average size of 800 to 2000 Da and thus can be analyzed with high sensitivity. These tryptic peptides are readily dissociated by collisional activation needed to obtain sequence informative data.

1.5.3 Proteomic mass spectrometry

Peptides generated from digestion are injected onto a microscale capillary high-performance liquid chromatography (HPLC) column that is online with the mass spectrometer. The peptides are eluted from these columns using a solvent gradient of increasing organic content so that the peptides species elute in order of their hydrophobicity (Steen and Mann 2004). Since the signal intensity of the mass spectrum is proportional to the analyte concentration, the peptides are eluted in as small a volume as possible by making using a microscale chromatographic column. Smaller diameter, 50-150 μ m inner diameter, chromatographic columns can be packed more uniformly. When the peptides arrive at the end of the column, they flow through the electrospray nebulizer needle. At the tip, the liquid is vaporized and the peptides are ionized by a strong electric potential. This process is called electrospray ionization (ESI) (Steen and Mann 2004).

Once the electrosprayed peptide ions enter the mass spectrometer via a vacuum system, they are guided by magnetic fields toward a detector which measures their mass to charge ratio (m/z ratio). Rather than determining the mass of molecules, mass spectrometry measures the mass to charge ratio whereby electrospray ionization generates ions with multiple charges such that the observed m/z value has to be multiplied by z in order to correct for the number of attached protons (which equals z) to calculate the molecular weight.

If tandem mass spectrometry is being used, the ionized peptides continue to travel through the instrument, allowing determination of primary structure (or sequence) information from the peptides. By coupling a second stage of mass spectrometry (tandem MS), a peptide ion can be isolated, energy imparted by collisions with an inert gas and this energy causes the peptide to break apart. The mass spectrum of the resulting fragment is then generated. If a fragment ion has one or more amino acid than another fragment, the m/z difference between the two corresponding peaks will equal the mass of the amino acid divided by the charge state (Xu and Ma 2006). The fragment ions containing the carboxyl terminus of the peptide are called y-ions and the fragment ions containing the amino terminus are b-ions. Throughout the chromatographic run the instrument will cycle through a sequence that consists of obtaining a mass spectrum followed by obtaining tandem mass spectra of the most abundant peaks (Watson 2007). A good spectrum will consist of a ladder of peaks of y-ions and a ladder of peaks of b-ions. Peptide sequence can be derived from the mass differences of adjacent peaks of the ladders (Xu and Ma 2006). Each tandem mass spectrum represents an independent and verifiable piece of data and therefore can be used to locate proteins in sequence databases (Eng, McCormack et al. 1994; Washburn, Wolters et al. 2001). However, many factors complicate the peptide sequence identification including absent b and y-ions, imperfect fragmentation and simultaneous fragmentation of two different peptides. Therefore software programs have been developed to aid in MS/MS peptide identification.

1.5.4 Proteomic data analysis

There are multiple ways to identify amino acid sequence from an MS/MS spectrum. Software programs exist that involve:

- a) database searching, which finds the best matching peptide from a protein sequence database (used only when a known protein database exists),
- b) *de novo* sequencing, which computes a peptide sequence directly from a spectrum,
- c) sequence tagging which combines the previous two methods by first conducting *de novo* sequencing to obtain short (usually 3 amino acid chain) sequence tags and then uses these tags to search a database, consensus searching, which combines several different programs to increase proteome the coverage (Xu and Ma 2006).

Database searching is the most common tool. There are many database search algorithms that use mathematical approaches to match sequence to spectrum. They include fragmentation models, spectral preprocessing and methods to match tandem mass spectra of peptides whose exact sequence may not be present in the database (Yates 1998). SEQUEST is a proprietary tandem mass spectrometry data analysis program used for protein identification (Eng, McCormack et al. 1994). This program compares collections of experimental tandem mass spectra to peptide sequences that have been generated from databases of protein sequences. A peptide's intact mass is known from an individual mass spectrum, SEQUEST uses this information to determine a set of candidate peptides (selecting candidates that are near the mass of the observed peptide ion) from a selected genomic database. For each possible candidate peptide, SEQUEST projects a theoretical tandem mass spectrum and compares it to the observed mass spectrum with the use of cross correlation (sum of the peaks that overlap between the theoretical and actual spectra). The theoretical mass spectrum that best matches the

experimental mass spectrum is reported as the best identification for this spectrum (Eng, McCormack et al. 1994).

1.6 Importance of the proteomics of bioremediation capable microbes

Bacterial strains such as *Geobacter uraniireducens* Rf4 and *Geobacter sulfurreducens* are useful for bioremediation purposes since they reductively precipitate a diverse range of heavy metals and radionuclides, including uranium by using acetate and other organic acids as the electron donor and it is possible to biostimulate these microbes *in-situ* to optimize their bioremediation potential (Anderson, Vrionis et al. 2003). Understanding the proteome of such microbes is a key component in deciphering the complex biological processes taking place. With this understanding comes the opportunity to further enhance these natural processes for the purposes of bioremediation.

1.6.1 Metaproteomics

Most post-genomic techniques have been limited to the study of pure cultures but these studies do not provide information about gene expression in the complex mixture of microorganisms found in the biosphere. Techniques must be developed that enable the study of microbial populations *in-situ* with respect to both natural habitat and community structure (Wilmes and Bond 2006). The genetic analysis of uncultured microorganisms (metagenomics) has resulted in many sequences of different microbial communities and have highlighted the extent of microbial functional diversity (Schloss and Handelsman 2003). With the availability of metagenomic sequences it is now possible to apply proteomics (metaproteomics) to these complex studies (Wilmes and Bond 2004). Examination of a microbial community through the use of proteomics will not only help to address the identification of an individual species within a complex mixture but also

allow for an assessment of each individual microbial role within a complex community of sediment microbes.

A wealth of information can be realized if proteins can be classified by function or biological pathways. Metaproteomics offer a unique opportunity to study sediment microbe relationships by examining changes in species composition and protein expression on a community level. These studies have the potential to combine systems biology and microbial ecology and bring to light individual microbial niches within a sediment community (Norbeck, Callister et al. 2006). In order to develop a systems level knowledge of molecular components within a cell that make bioremediation possible, there must be a comprehensive characterization of the cellular proteins and how their abundance, location and modification state respond to changing conditions. This task has been historically difficult due to the complex environment in which microbial communities tend to exist.

Chapter 2

Extraction of Sediment Microbial Protein

2.1 Introduction

Characterizing sediment microbes capable of bioremediation in terms of protein expression provides information needed to enhance microbial nutrient factors for reducing heavy metals and to better understand the unique biological pathways that make these processes possible. Prior to mass spectrometric analysis the microbes must be removed from the sediment and the proteins extracted and digested into peptides. Initial removal of microbes from the sediment particles has proven to be a significant problem. Therefore, it's useful to probe methods of direct *in-situ* lysis of microbes followed by removal of protein from the sediment. Many extraction techniques have been tested on sediment bacteria genomic DNA extraction, cell enumeration and metabolic studies but a protocol specifically designed for proteomic studies in a high throughput laboratory setting is needed and investigated herein.

2.2 Great plate count anomaly

Attempting to extract and analyze the full complement of bacterial protein from any particular sediment has a wide array of difficulties. First and foremost is the general need to cultivate a microorganism in order to study its physiological characteristics and from these data assemble a more complete picture of its ecological properties. Only a small fraction (0.1% - 10%) of natural microbial communities have been successfully cultivated (Rogers, Moorman et al. 2007). Studies have been hampered by enrichment bias where standard cultivating techniques select for easily culturable organisms giving rise to a distorted representation of natural microbial communities (Wilmes and Bond

2006). Using epifluorescence microscopy it has been shown that one gram of soil may contain more than 10 billion prokaryotic organisms, whereas plate counts are often 100 to 1000 fold fewer, a phenomenon known as the Great Plate Count Anomaly (Staley and Konopka 1985). There are a few explanations for this anomaly including the fact that species that would otherwise be "culturable" may fail to grow because their growth state in nature, such as dormancy, prevents adjustment to conditions found in the medium used for the plate counts (Colwell 2000). Also, many of the microbial species that dominate in natural settings are not adapted for growth in media containing high concentrations of complex organic carbon (Connon and Giovannoni 2002).

This anomaly raises the need to extract a full representation of microbial community proteins from the sediments in reasonably large quantities. This task has been very difficult for a number of reasons that need to be individual analysis to develop an efficient protocol for extraction of geomicrobes for proteomic analysis.

2.3 Biofilms

Bacteria living in sediments develop biofilms in order to support growth and survival in organized communities (Jenkinson and Lappin-Scott 2001). Biofilms are primarily composed of extracellular polymeric substances (EPS) that facilitate adhesion to submerged surfaces and provide a structural matrix. This matrix is organized to make use of all available nutrients. Generally matrices are composed of diverse substances, including polysaccharides, proteins, nucleic acids, lipids and humic substances (Tsuneda, Aikawa et al. 2003). Matrices typically form at lower temperatures and in the presence of salts and are rich in hexoses like glucose and galactose (Sutherland 2001). For these reasons, any single biofilm formed under any specific set of conditions may well be

unique to that single environment. Since an enormous number of microbes are capable of forming biofilms, the structure and chemistry of individual biofilms is anything but static (Sutherland 2001). Mixtures of microbial species, such as those found in sediment, produce varying proportions of different polysaccharides synthesized within the biofilms which give rise to a vast combination of compositions (Sutherland 2001). The structures that are formed depend on a large variety of parameters including: species, temperature, flow conditions, pH and presence of salts (McLandsborough, Rodriguez et al. 2006). Polysaccharides, the predominant biomolecule in biofilm matrices, are long, thin molecular chains and can associate in many ways including electrostatic interactions, hydrogen bonding and ionic interactions (Mayer, Moritz et al. 1999). Flocculant formations or networks are formed by one macromolecule fitting into another resulting in a poorly soluble substance. Increased viscosity can occur to the polysaccharides when ions are present and interact with carboxylic acid groups on the EPS (Sutherland 2001).

A bacterial biofilm matrix can impact analyses of sediment microbes by making them less accessible for sample preparation (Bockelmann, Szewzyk et al. 2003). In order to remove intact bacterial cells from sediment, it is essential to break apart the EPS; however, the composition of bacterial biofilms impedes a standard protocol for doing so. Also, attempting to extract intact bacterial cells demands a protocol harsh enough to remove the biofilm but gentle enough to allow the bacterial cell wall membrane to remain intact. This could create a situation where some cells are lysing while others are not, resulting in a false representation of the microbial community.

2.4 Properties of sediment

Sediment is a heterogeneous mixture very similar to soil but sediment is classified as particulate that can be transported by fluid flow which is eventually deposited at the bed or bottom of a body of water (Nichols 1999). It is essential to understand how sediment characteristics control the activity of microorganisms and the types of interactions they have with sediment in order to study removal. The biology of sediment and soil is extremely complex and results from multiple interactions between physical structure, interface phenomena, soil biota composition and activity, population dynamics, chemical composition, time, and environmental conditions (Arino 2002). Therefore, interactions will vary dependant upon many factors. A few of the most important include size of the sediment particles, composition and charge.

2.4.1 Particle size

Inorganic sediment particles can be classified into three major groups according to their size: sand, silt and clay. The proportions of these in any one soil or sediment sample determine the soil texture. According the U.S. Department of Agriculture, sand is 0.05mm to 2.0mm, silt is 0.002mm to 0.5mm and clay is less than 0.002mm. The number of soil particles per gram can range from 90 (pure sand) to 90 billion (pure clay) (Dirk van Elsas 1997). To emphasize the high degree of physical heterogeneity present in sediment, the surface area of sediment particles can range from 11 cm² g⁻¹ to 8 million cm² g⁻¹ (Dirk van Elsas 1997). The many biogeochemical processes that occur within sediment create a mosaic of microsites and gradients within the environment. Basically every sediment aggregate is a habitat for some type of microorganism. Generally half of a sediment aggregate is made up of void spaces or pores, connected by pathways. The

relationship between these void spaces and pathways are what make up the sediment microorganism niche. Water within these channels also transport a significant amount of freely motile bacteria (Corapcioglu and Haridas 1984). When considering protein adsorption, clay is the most important size fraction due to increased surface area. Also, the finer the fraction the greater swelling properties and the less crystallinity (Robert, Hardy et al. 1991).

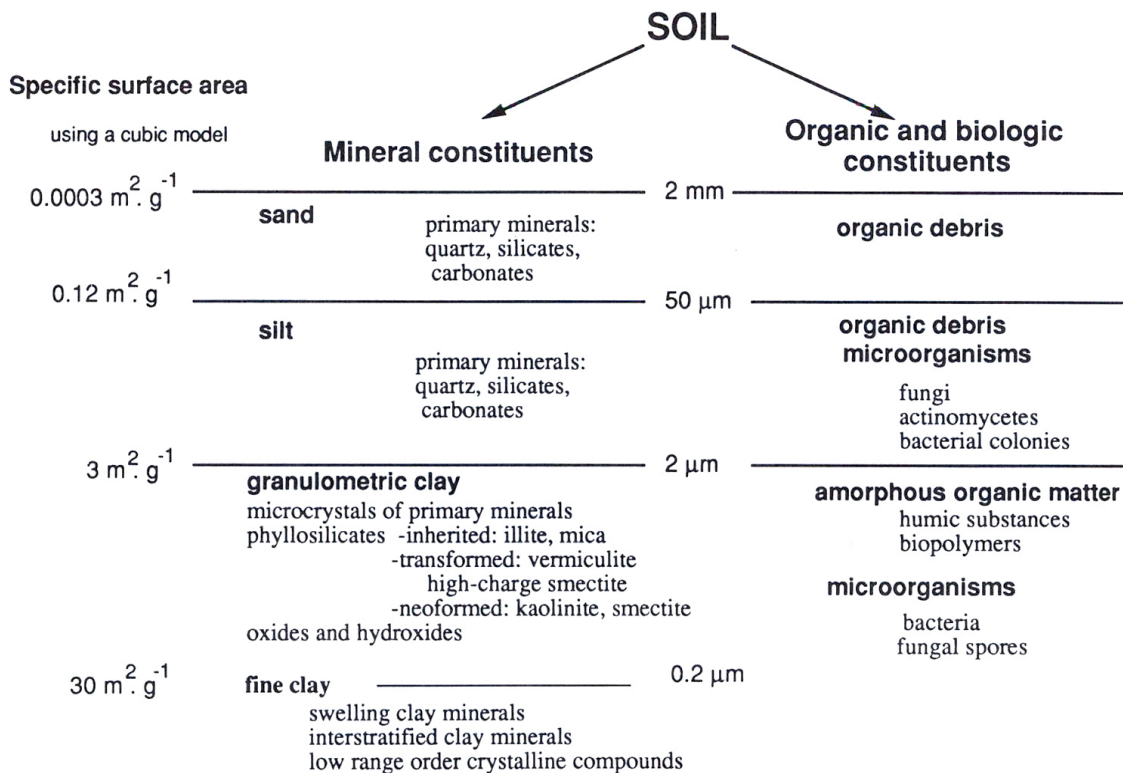


Figure 2.1 Size fractionation of soil constituents (Stotsky 1992)

2.4.2 Composition

Sediments consist of four major components including two solid portions (organic and inorganic), liquid and gas phases. The solid phase is composed of minerals (containing particles of various sizes, shapes and composition) and an organic component composed of live organisms as well as decomposing plant and animal matter. The liquid

phase is composed of water which fills a portion of open spaces between the solid phases, and contains dissolved solutes from minerals and is largely varied from sediment location to location. The gaseous phase occupies the pore space that is not filled with the liquid phase and changes dramatically over time. The relationships among the sediment constituents continually change depending on temperature, pressure and light (Jury 2004).

The sand and silt fraction of sediment are composed of primary minerals such as quartz and feldspars. Depending on the size and shape of primary minerals, their arrangement can result in different overall porosities and range of pore spaces. This fraction represents the “inert” skeleton of the soil ($>2\mu\text{m}$ particle size). The sediment fraction that contributes most to sorption is clay (colloidal level) due to the high surface area ($<2\mu\text{m}$ particle size) and physical properties. This fraction represents the active fraction and clay minerals include (1) kaoline group (kaolinite, illite, dickite, halloysite and nacrite), (2) smectite group (montmorillonite, nontronite and saponite) and (3) chlorite group and (4) illite group (McCarthy 2002). A portion of the $<2\mu\text{m}$ particle size group consist of micro-divided primary minerals (quartz, feldspar, calcite) but it's important to note that they do not have the same structure or properties of clay minerals. The mineralogical clays have a specific structure that lends itself to chemical and physical properties that increase adsorption (Stotsky 1992).

X-ray diffraction is a way to identify sediment components since “every crystalline substance gives a pattern; the same substance always gives the same pattern; and in a mixtures of substances each produces it's pattern independent of the others”

(Hull 1919). The x-ray diffraction of a substance is unique and can therefore be used for the identification of an unknown substance.

2.4.3 Charge

The solid phase of sediment can exhibit both fixed charges and variable charges. Mineral clay components tend to be of a fixed charge and metal oxides tend to be variable. The variable charge component is what makes bioremediation possible as biodegradation is an electron transfer process. Certain microbial enzymes catalyze electron transfer to gain biological energy. Organic compounds such as acetate or sediment contaminants such as hydrocarbons act as electron donors for the microbes. (WDNR 1994). These electrons are moved through the microbial respiratory or electron chains (metabolic pathways) to recover the chemical energy. This can lead to metal reduction, for example at the end of this process soluble metals, such as Uranium (VI) can then accept the electrons reducing it to Uranium (IV). In biodegradation, complex organic molecules are converted to simpler compounds, ultimately, to carbon dioxide or methane and water (Diels 2007). *In-situ* bioremediation relies on the ability of microbes, either attached to soil particles or suspended in the pore water, to either convert contaminants into harmless molecules (carbon dioxide and water), or to immobilize them into non-soluble and stable forms such as what is taking place at the Rifle site. Electric charge on sediment organic matter can change with pH and various protein functional groups dissociate at different pH values. At high pH, some functional groups will lose a proton and become negatively charged while others will associate with a proton at low pH and become positively charged (Barrow 2005). Even though the solid phase of sediments is a heterogeneous mixture of minerals with different charge behavior,

electrophoresis reveals that sediment particles are generally negatively charged in the pH range 3-9. Iron and aluminum oxides, which carry a net positive charge at low pH, form aggregates with negatively charged minerals and end up carrying a net negative charge (Borgnino, Avena et al. 2006). To test methods for disassociating microbial protein from sediment particles, various pH values should be considered for a range of protein functional groups. The sediment composition should also be considered since ion adsorption and exchange are important processes that take place within sediments and can have a significant effect on the efficiency of protein extraction and the blocking of protein adsorption sites.

2.5 Extraction techniques

There are two ways to attempt the extraction of protein from sediment microbes:

- 1) Indirect extraction: removal of intact bacterial cells from the sediment followed by lysis (apart from the sediment) and followed by digestion, or
- 2) Direct extraction: *in-situ* lysis of bacterial cells (release of protein from bacteria while still on the sediment) followed by protein removal and digestion.

2.5.1 Analysis of the indirect extraction technique

2.5.1.1 Microorganism adsorption to sediment surfaces

The composition of DNA extracted from soil and sediment indicates microbial populations may be comprised of over 10,000 species per gram of material (Torsvik, Goksoyr et al. 1990). To release microorganisms from sediment for the purpose of studying the process of biodegradation, it is important to understand sediment characteristics and the interactions they have with microbes. Microorganisms living in sediment have various sorptive interactions that can impede removal because the

environment is dominated by heterogeneous particles of both large and small surface areas and interactions depend on the texture and mineralogy of the particles. These particles also exhibit permanent and variable charges depending on the soil fraction or texture (see section 2.4). Microorganisms exist in multiple interactions with sediment including within pore spaces, attached by biofilms (see section 2.3) and electrostatically. A disadvantage of indirect cell extraction is that if the procedure is inefficient, the protein may be less representative of the entire microbial community with a potential for bias towards a particular phylogenetic group. Different bacterial groups adhere more or less strongly to soil particles (Prieme, Sitaula et al. 1996) which might bias the picture of the composition of the microbial community in the sample. Research suggests that most microorganisms in sediment exist in a sorbed state (Stolp 1988). Because the degree of sorption is greatly related to the surface area and surface charge properties of the particles, the colloidal fraction is generally the most important soil component with respect to sorptive interaction (Marshall, Stout et al. 1971). This is emphasized by Grey et al. (1968) who found that 60% of bacteria in sandy sediment are located on the small particles that represent only 15% of the surface area. They calculated that only 0.02% of the surface area of the sand particles were colonized (Gray 1968).

The methods employed to study sorptive interactions include counting procedures, microscopy, electrophoretic techniques and particle size analysis. From these studies it has been found that the ability of different soils and sediments vary in their ability to sorb bacteria depending on the mechanical composition of the sediment and the species and growth conditions of the microorganisms. In general, the extent of adsorption of bacterial cells is greatest for clays and least for sands (Marshall 1971).

2.5.1.2 Pore spaces

In sediment, the habitat for microorganisms is the solid phase, which is spatially arranged in a discontinuous pattern of various sizes and shapes that are filled with water or air. Bacteria exist within the tiny complex aggregates of the sediment, which can mask the cells from extraction. Sediment and soil aggregates are defined by Martin et al. (1955) as “a naturally occurring cluster or group of soil particles in which the forces holding the particles together are much stronger than the forces between adjacent aggregates (Martin and Gottlieb 1955). Bacteria can exist within aggregates or between them and depending upon the level of porosity in a particular sediment, can be very difficult to release.

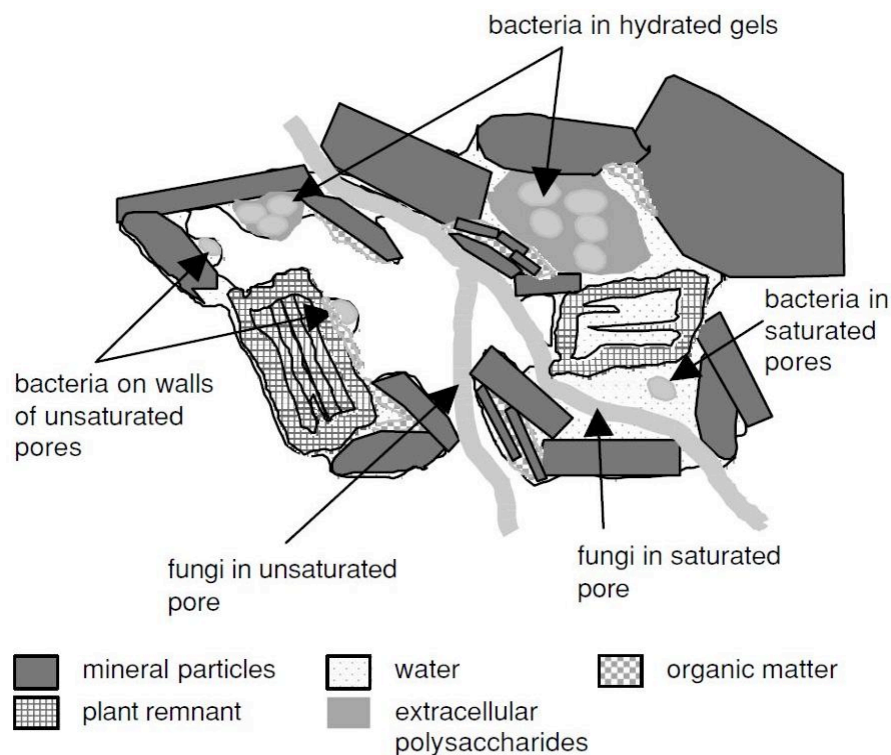


Figure 2.2 Representation of sediment microbe habitat (Huang 2002)

2.5.1.3 Analysis of previous research

In order to separate bacterial cells from sediment particles, the sediment must be dispersed followed by separation of the bacterial cells from the sediment. Bacterial abundance is higher in inner sediment aggregates than on the outer layer of aggregate, and the composition of bacterial communities varies between these two sites (Hattori 1973). Strong binding within aggregates and between cells and surfaces are formed from a variety of binding mechanisms such as the extracellular polysaccharides in biofilms. Therefore it is important to disperse and homogenize the sediment prior to extraction to achieve a true representation of indigenous soil bacteria. This requires that cells be dislodged from particle surfaces and released from within the aggregates. Dispersion can either be chemical, physical or a combination of both. Examples of physical dispersion include sonication (bombardment of the sediment particles with high intensity ultrasound waves), Waring blender, mortar and pestle, and shaking with glass beads (Ellery and Schleyer 1984; Ramsay 1984; Schallenberg and Kalff 1993; Danovaro, Fabiano et al. 1994; Kepner and Pratt 1994; Lindahl and Bakken 1995; Lindahl 1996; Mayr, Winding et al. 1999; Whiteley, Griffiths et al. 2003). Chemical dispersion is used to interact with particle surfaces in order to release adsorbed objects. Chemical treatments include salts to weaken the biofilm matrix by screening out crosslinking electrostatic interactions, chelating agents to reduce EPS cohesiveness (Tris buffer, sodium pyrophosphate or sodium-charged ion exchange resins) and by exchanging polyvalent cations bound to negatively charged clay particles for monovalent cations thereby reducing electrostatic interactions between clay particles and negatively charged soil bacteria, surfactants to disrupt hydrophobic interactions and urea for reducing hydrogen bonding in the biofilm

(Faegri, Torsvik et al. 1977; Niepold, Conrad et al. 1979; Bakken 1985; Balkwill and Ghiorse 1985; Macdonald 1986; Macdonald 1986; Yu, Dodds et al. 1995; Xavier, Piciooreanu et al. 2005).

Following dispersion, the released bacterial cells must be separated from the sediment. Traditionally this has been achieved by centrifugation. During low speed centrifugation, detached cells and sediment particles are separated according to the differences in their sedimentation velocities (Mayr, Winding et al. 1999). High speed density gradient centrifugation can be used by employing a high density material such as Nycodenz (Axis-Shield Oslo, Norway) which has a density of 1.3 g ml^{-1} . Once dispersion is complete sediment particles can be sedimented through the gradient and bacteria form a band within the gradient and be collected (Lindahl and Bakken 1995; Lindahl 1996).

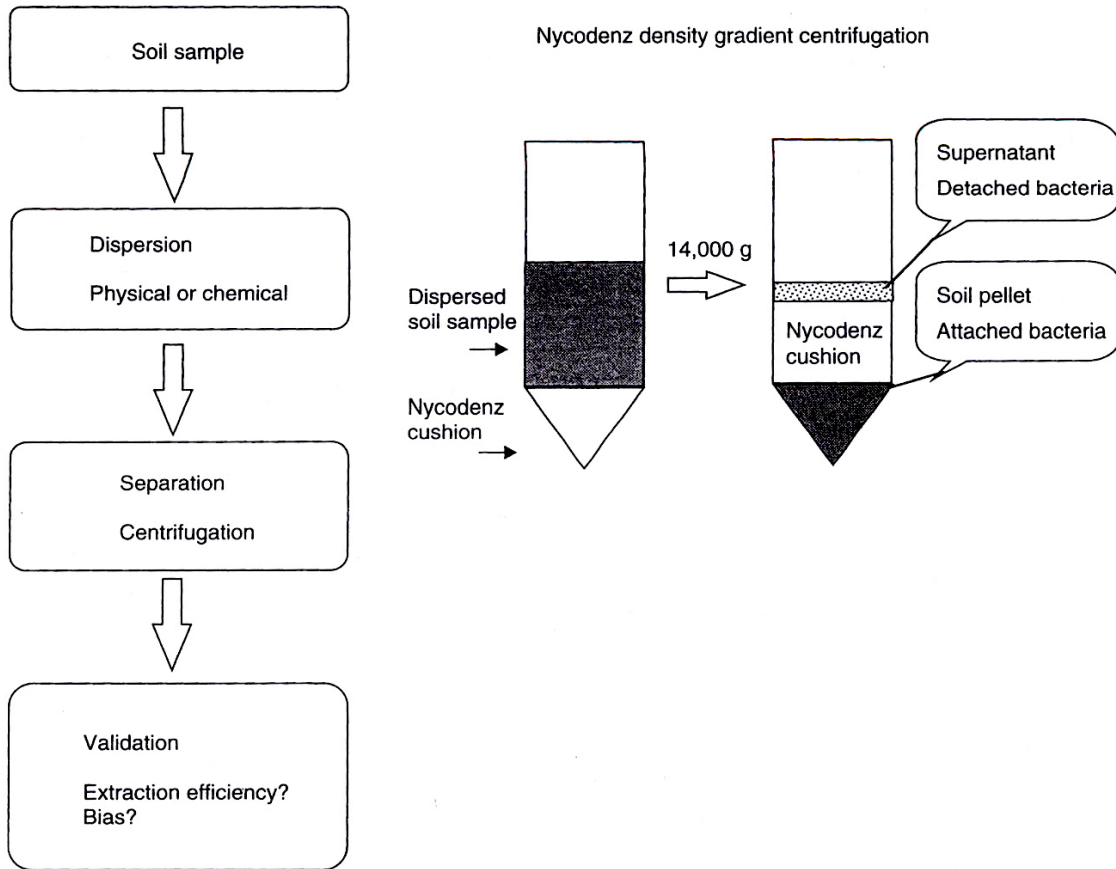


Figure 2.3 Nycodenz density gradient scheme (Dirk van Elsas 1997)

Despite a multitude of efforts optimizing the removal of intact bacterial cells from sediment, typical extraction efficiencies still only range from 20-30% for clay loams and 30-50% for peat soil (Dirk van Elsas 1997)

2.5.1.4 Discussion

Studies have shown that different microorganisms adsorb onto soils, clays, ion-exchange resins and glass at varying rates (Dirk van Elsas 1997) and some microbes lyse easily while some are more robust. Bacteria also exist within tiny pore spaces and are strongly attached by biofilms. Therefore, it's very difficult to remove the full compliment of intact bacterial cells within a sediment community without biasing for easily detached, robust bacterial cells. No indirect extraction technique is ideal since gentle disruption

does not remove an adequate number of cells and harsh disruption leads to damaging the cell.

2.5.2 Analysis of the direct extraction technique

2.5.2.1 Protein adsorption to sediment surfaces

The bacterial cell is surrounded by a lipid membrane which encloses the cell and acts as a barrier to hold proteins and other essential cellular compounds but once lyses occurs, microbial proteins are released. The disadvantages of direct lysis of the cell wall on the sediment (for subsequent removal) include differences in cell properties and the extent to which soil structures protect cells from lysis treatments giving rise to a biased representation of easily lysed bacteria. Also, the liberated protein can become adsorbed to sediment colloids leading to an underestimation of protein (Frostegard, Courtois et al. 1999). Biomolecules such as protein tend to undergo serious interactions with surfaces from reversible binding to irreversible adsorption. The simplest model for adsorption of proteins onto a surface includes (1) the transport of the protein to the surface (2) interaction and attachment of the protein to the surface (this step may involve a rearrangement of protein structure but is may still be capable of desorbing) (3) increased residence time the adsorbed protein may relax into a steady state conformation which is irreversibly retained on the surface (Norde 1995).

The interaction of protein with surfaces such as sediment is a complex phenomenon that can be attributed to the diverse range of physiochemical properties of both the sediment and the bacterial protein. The physiochemical characters of the protein such as isoelectric point (pI), the flexibility of the polypeptide chains, charge, hydrophobicity and the acid/base characters (Rechendorff 2006) are all highly variable,

as are the surface properties of sediment colloid particles (clay minerals and humic substances) which provide a wide range of polar and non-polar adsorption sites. Also each amino acid has side chains that differ in charge (negative, neutral or positive) as well as a hydrophobicity scale (hydrophobic nonpolar to hydrophilic polar) (Creighton 1993). No type of molecular interaction is unimportant to the adsorption process (Norde 1995). Adsorption of protein involves many dynamic steps and can be divided into the following major contributions (A) hydrophobic/hydrophilic interactions (B) structural rearrangements or structural stability of the protein (C) van der Waals interactions and (D) electrostatic interactions between the protein molecules and the adsorbent (Bremer, Duval et al. 2004).

2.5.2.2 Hydrophobic/hydrophilic interactions

Sediment surfaces have both hydrophobic and hydrophilic characters. Clay fractions tend to be more hydrophilic and when in contact with a hydrophilic protein they will tend to retain a hydrated layer between them. Silt and sand fractions are more hydrophobic (due to the composition which is more susceptible to developing a wax-like coating due to the production of complex organic acids during decomposition of organic matter) (Doerr, Shakesby et al. 2000). When two hydrophobic entities interact they repel water from their surface of interaction. This dehydration of a hydrophobic surface contributes to the overall energy of adsorption much more than that of electrostatic interaction between the protein and the sorbent under most conditions (Elgersma, Lyklema et al. 1991) Thus in general, proteins adsorb on hydrophobic surfaces even if the protein and sorbent repel each other electrostatically. On hydrophilic surfaces the hard proteins (a strong internal coherence) adsorb only if electrostatically attracted (Arai and

Norde 1990). However, it has been shown that proteins with less stable native structures tend to adsorb on electrostatically repelling, hydrophilic surfaces and thus other factors must favor the adsorption process to an extent that outweighs the opposing contributions from electrostatic repulsion (Norde and Favier 1992). Therefore, experimental data points to the notion that structural rearrangements are the driving force for protein adsorption in this case (Kondo, Oku et al. 1991).

2.5.2.3 Structural rearrangements

Sediment particles can be surrounded by water films which affect protein adsorption through several interactions including enthalpic forces or entropic effects that promote conformational modifications of the adsorbed protein (Leita, Fornasier et al. 2006). As noted before, proteins with a strong internal coherence are less affected by the structural rearrangement adsorption mechanism and therefore adsorption is primarily governed by electrostatic interaction and dehydration of hydrophobic areas of the sorbent and/or protein molecule (Norde and Anusiem 1992), (Norde and Favier 1992). For protein with a weaker internal coherence, experimental data points to structural changes upon adsorption such as the decrease in α -helix content of the protein upon adsorption (Kondo, Oku et al. 1991) (McMillin and Walton 1974). This reduction causes an increased rotational mobility along the polypeptide chain, leading to larger conformational entropy of the protein (the dominant driving force for adsorption) (Andrade and Hlady 1986).

2.5.2.4 van der Waals interactions

The van der Waals force is a transient, weak electrical attraction of one atom for another and van der Waals attractions exist because every atom has an electron cloud that

can fluctuate, yielding a temporary electric dipole. The transient dipole in one atom can induce a complementary dipole in another atom, 0. provided the two atoms are quite close (Branden 1991). These interactions differ from covalent and ionic bonding in that they are caused by correlations in the fluctuating polarizations of nearby particles (a consequence of quantum dynamics). The van der Waals interactions are typically short ranged as opposed to electrostatic interactions. Zhdanov et al. (2001) have found that hydrocarbons can reduce the van der Waals forces in protein-surface interactions (Zhdanov and Kasemo 2001).

2.5.2.5 Electrostatic interactions

Sediment minerals possess a net negative electrical charge on their surface. Positively charged proteins with isoelectric point (pI) values well above the buffer pH may become electrostatically bound to (mainly) clay particles due to coulombic attraction. Since the amount of positive charge on the protein increases as the pH decreases (due to protonation), higher adsorption would be expected as the pH decreases below the pI of the protein. Proteins with a pI near the buffer pH of 7.0 show only partial adsorption, while proteins with a pI well below pH 7.0 show increasing recovery as a result of the coulombic repulsion between the anionic protein and the negatively charged sediment particles. The charge localization also plays a role in protein adsorption. Protein configurations in which numerous positive protein charges are close to the adsorbent, produce higher binding (Yao and Lenhoff 2004) so flexibility of the protein plays a role the strength of electrostatic interactions.

Because of the long-ranged nature of the electrostatic interactions, protein and (and individual amino acids) may be guided into a unique orientation when approaching

the oppositely charged surface. This suggest that when binding sediment protein adsorption sites, the pH of the binder should be kept below the pI to ensure a maximal positive charge and increase the electrostatic attraction to the negatively charged sediment. When lysing the bacterial cell the pH should be increased to induce coulombic repulsion between the released protein and sediment particles.

2.5.2.6 Sediment mineral composition

While, a low pH (higher positive charge) should result in stronger protein adsorption to negatively charged sediment, results have shown that adsorption by smectite clays is greatest at or near the pI of protein (Bollag 1990). Explanations for this phenomenon include a decrease in positive charges associated with the protein require higher uptake of the protein to satisfy the negative charges on the smectite, also as protein approaches the pI the structure becomes less compact leading to higher adsorption (Albert and Harter 1973). Proteins have been shown to adsorb on vermiculite, illite and kaolinite but at much lower amounts than smectite. Protein adsorbs to smectite > vermiculite = illite > kaolinite. Protein appeared to be intercalated by smectite but adsorption was limited to the external surfaces of the other minerals (Albert and Harter 1973). For kaolinite the adsorption maximum is achieved at several pH units below pI and is primarily due to ion exchange.

2.5.2.7 Discussion

In protein-surface interactions, not only are the interactions determined by the physical state of the material and the protein surface but also the intimate solution environment. Factors that should be considered when studying protein adsorption include properties of the sediment surface, protein properties, surface charge, surface roughness,

pH, ionic strength of the solution, temperature, surface elemental composition and surface energetics. (Israelachvili 1992).

2.6 Direct extraction with prior blocking of protein adsorption sites

Previous research suggests that indirect methods of bacteria removal from sediment is ineffective and also leads to a bias representation of the microbial community. Indirect lysis is deemed to be the most effective method. However, protein adsorption to sediment is a complex problem. Two approaches can be taken when trying to minimize protein adsorption to sediment particles. The first includes the modification of the sediment to produce an inert nonadsorptive surface and the other is providing certain electrophoretic conditions that minimize electrostatic interactions. It is hypothesized that a combination of both approaches can be used to reduce protein adsorption onto sediment following indirect lysis.

2.6.1 Modification of the sediment to produce a non-adsorptive surface

In the pH range of 3-7, the net charge of sediment particles is negative (Bowles 1979). In the same pH range, polar positive amino acids (histidine, arginine and lysine) are below their isoelectric points (pI) (7.6, 10.75 and 9.6 respectively) and therefore strongly positively charged (due to protonation). Under these conditions the polar positive amino acids are strongly electrostatically attracted to the sediment. Therefore it is hypothesized that in order to avoid the protein loss encountered from direct lysis of protein on sediment (due to released protein adsorption), the sediment can be pre-treated (prior to lysis) with polar positive amino acids in the pH range of 3-7. These amino acids will strongly bind to the electrostatic adsorption sites on the sediment,. Once cell lysis takes place the protein will be free for extraction instead of adsorbing onto the sediment.

Individual amino acids are much too small to be detected on the mass spectrometer so they will not interfere with analysis.

Other potential binding agents include the use of amines. Verzola et al. investigated monoamines (triethylamine, triethanolamine, ethylamine), diamines (putrescine, cadaverine and hexamethonium bromide), followed by oligoamines (spermidine, spermine and tetraethylenepentamine (TEPA)). They found mono and di amines to be poor binders but oligoamines were effective at quenching protein surface interaction with silica tubing. Spermine and TEPA were the most effective binders at sub-milimolar ranges in a Tris-acetate buffer at pH 5.0 (Verzola, Gelfi et al. 2000).

Non-fat milk and casein have been used in a number of applications for blocking non-specific binding sites. Examples include western blotting, filter passivation and chromatography. These binders can also be tested on sediment but since they are themselves proteins, they could interfere with downstream analysis.

Conditions during the blocking of binding sites must be optimized to enhance electrostatic interactions between the binder and the sediment. Conditions such as pH, temperature, binding time and binder concentration can all be tested. Once non-specific binding sites have been blocked the conditions must be reversed directly prior to lysis and release of sediment community proteins. The conditions during lysis must favor reduced electrostatic interaction. Since binding and desorption takes place over time, reversal of these conditions will not release the binder in the matter of minutes it takes to lyse the cells. Even if partial reversal does take place, this means the binding site was weak and proteins are not likely to bind there anyway. Prior to blocking non-specific binding sites, the sediment aggregates must be broken and dispersed to release microorganisms.

In order to reduce the biased representation of easily lysed bacteria, lysis can take place on a Barocycler, subjecting the entire sample to 35,000 psi over a number of cycles to ensure complete lysis of all bacteria. However, for experimental purposes and to keep protein masses constant, known quantities will be added to sediment for the quantification of extracted protein.

2.6.2 Development of a protein desorption buffer

Once binding sites have been blocked and the sediment is rendered inert, the microorganisms within the sediment must be lysed and the protein then needs to be separated from the sediment. Many chemicals can help in the efficiency of protein removal, but the choices must be amenable to clean-up for mass spectrometry measurement.

Stable proteins have been shown to adsorb at a lower rate than denatured or partially denatured proteins, attributed to decreased surface area of the protein giving rise to less chance for adsorption sites (Wendorf, Radke et al. 2004). With increasing denaturation, the interaction energy in the adsorbed state is increased and the probability for desorption is decreased (Andrade, Hlady et al. 1992). Sugars have been shown to stabilize proteins in solution possibly resulting from preferential exclusion of sugars from the protein surface (Timasheff 1998). Proteins in a sugar solution show reduced denaturability and improved stabilization leading to less adsorption (Kato and Yutani 1988; Mcguire, Wahlgren et al. 1995). Wendorf et al. (2004) tested bovine serum albumin (BSA) adsorption to a hydrophilic and a hydrophobic surface in the presence of sugars. They found that at the same sugar concentration the ability of sugars to reduce protein adsorption followed the trend: trisaccharides > disaccharides > 6-carbon polyols >

monosaccharides. However, trisaccharides have low solubility and could only be tested in low concentrations. The most effective sugar at reducing protein adsorption was 1.5M sucrose (Wendorf, Radke et al. 2004). Sugars can clog mass spectrometer columns are difficult to clean up at the peptide level through solid phase extraction. Therefore, a sugar solution would have to be cleaned out of the sample on the protein level with the use of centrifugal filtration devices with a 3K molecular weight cut off to ensure optimal protein recovery. Many wash steps would have to be involved since sugars can clog mass spectrometer columns. Since high concentrations of sugars can be problematic, a low concentration of trisaccharides seems to be the best choice for proteomic studies.

Salts can weaken electrostatic interaction between proteins and surfaces by associating with charged groups and give a dispersing effect. They can also weaken the biofilm matrix by screening out crosslinking electrostatic interactions. Kondo et al. showed the effect of ionic strength on the adsorption of human hemoglobin (HHb) on ultrafine silica particles at pH 7.0 and 8.0 at 30°C. The adsorption amount decreased significantly with increasing ionic strength of 0.01M, 0.02M and 0.1M NaCl (Kondo and Fukuda 1998). Salts however, favor retention by increasing hydrophobic interaction. This problem can be overcome by the addition of the dilute chelating agent EGTA (Ethyleneglycol Bis(2-Aminoethyl Ether)-N,N,N',N' Tetraacetic Acid) which causes a small conformational change that can mask hydrophobic surfaces of protein. EGTA has also been found to reduce biofilm attachment by which the chelant removed essential calcium from the biofilm causing the biofilm to detach (Turakhia, Cooksey et al. 1983).

Lower protein absorbance was found at higher pH ranges due to deprotonation of the protein enhancing coulombic repulsion with the negatively charged silica particles

(Kondo and Fukuda 1998). Since sediment is negatively charged, a desorption buffer should be above the typical pI of proteins. It is important to note that extreme pH changes can affect both the chemical and physical stability of proteins. While there is an increase in protein desorption at a high pH, many unfavorable reactions can occur such as peptide bond hydrolysis, deamidation and double bond formation in alkaline conditions (Wei 1999). Therefore, the alkaline pH should not be extreme. Kondo et al. also tested the effects of temperature on protein adsorption and found that the lower the temperature the lower the entropic driving forces caused by conformational changes during adsorption (Kondo, Urabe et al. 1996). A low temperature after lysis is also important to reduce the endogenous proteolytic activity caused by released natural enzymes within a cell.

Displacement agents can be used to desorb proteins attached to a surface by sequential detachment of segments in favor of the attachment of a newly arriving molecule. This can be visualized as one molecule stripping off and replacing another. Norde et al. used a solution of 0.05% morpholine, pH 8.5 to effectively displace adsorbed protein and subsequently cleaned the solution via dialysis or molecular filtration (Norde and Anusiem 1992).

Chaotropic agents reduce hydrogen bonding and reduce hydrophobic interactions. However, chaotrophs can denature protein rendering them less stable and more prone to adsorption. Therefore, chaotrophs can be added to the desorption buffer in concentrations lower than those typical of denaturataion concentrations. Chaotrophic agents in these concentrations have also been noted to show a beneficial effect on protein folding (Orsini and Goldberg 1978).

Since hydrophobic interactions play a role in protein adsorption, Tilton et al. tested the addition of alcohols to decrease protein adsorption. They found that methanol impaired protein/surface interaction.

Wei et al. found that buffer choice and concentration can drastically affect protein adsorption. Tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCL) was compared to phosphate buffered saline (PBS), both at pH 7.4 and at concentrations of 0mM, 10mM and 100mM. 100mM PBS greatly reduced protein adsorption at a liquid/solid interface and it was suspected that this is due to phosphate ions adsorbing on surfaces competing with protein adsorption and more than one type of phosphate ion existing in the solution (Wei, Kaewtathip et al. 2009). However, Larsericsdotter et al. (2005) compared protein adsorption between the buffers 10mM (N-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]) Hepes at pH 7.0, 50mM ammonium bicarbonate at pH 8.0 and 10mM phosphate buffer at pH 7.0. They found that a larger amount of the protein BSA (bovine serum albumin) adsorbed onto silica particles in the phosphate buffer than in the Hepes buffer and ammonium bicarbonate buffer. This observation was explained by BSA adsorbing in a more compact conformation when dissolved in a phosphate buffer which is ascribed to the relatively high efficiency of phosphate to bind to BSA and inhibit structurally unfavorable electrostatic repulsion (Larsericsdotter, Oscarsson et al. 2005).

Surfactants (or surface active agents) have a hydrophobic tail and a hydrophilic head. They are an effective way to desorb and solubilize proteins. Due to their amphiphilic properties, surfactants aggregate in solution to form micelles where the hydrophilic heads remain in contact with the solvent thus, sequestering the hydrophobic

tails. When surfactants are present in concentrations above the CMC (critical micelle concentration), they act as emulsifiers which allow normally insoluble proteins to incorporate into the micelle core. The micelles are themselves dissolved due to the favorable interactions between the hydrophilic head group and the solvent. Detergents are a very useful tool for solubilizing protein but they can pose significant difficulties as well. They are classified into 4 groups including: 1) Non-ionic – gentle detergents used to solubilize proteins while maintaining native subunit structures. 2) Anionic – negatively charged, strong detergents that can disrupt cell membranes and denature proteins. They must be completely removed since they can interfere with charge based analytical systems such as mass spectrometers. An anionic detergent such as SDS (sodium dodecyl sulfate) is one of the best detergents for desorbing proteins but is difficult to remove. 3) Cationic – positively charged, strong detergents that can be used to neutralize a negatively charged surface. 4) Zwitterionic - electrically neutral detergent that protects the native structure of protein and prevent aggregation. Detergents can be removed on the peptide level with solid phase extraction.

2.7 Discussion

Six test steps are included in the development of a protocol for the removal of protein from sediment:

- 1) Blocking sediment binding sites - various types of binding material can be tested for the efficacy of blocking binding sites. Also, various conditions that induce the best environment for blocking adsorption sites on sediment can be tested. These conditions include various pH ranges (below the pI of the binder), various temperatures for binding (increased temperature should increase binding but need

- to keep in mind the microorganisms that need to be preserved) and various times for binding.
- 2) Reverse high binding conditions - once sediment has the adsorption sites blocked, the conditions for optimal binding need to be reversed prior to lysis. This step needs to be done quickly and under cool temperatures. This reversal of conditions should keep the sites blocked since adsorption and desorption take place on a time scale. While some sites will instantaneously be reversed, these sites will likely not be high binding sites of released protein. Along with the reversal of binding conditions, a desorption buffer needs to be added to the sediment prior to lysis. Chemicals and conditions used to enhance protein desorption include salts, detergents, higher pH, lower temperature and sugar.
 - 3) Lysis - sediment samples need to be added to barocycler pulse tubes and subjected to high pressure for microbial lysis and protein release (for controlled experimentation purposes, this step can be simulated by the addition of known quantities of lysed bacterial protein).
 - 4) Separation of protein from sediment - once the sediment microbes have been lysed, the entire sample can be subjected to low speed centrifugation and the sediment washed with the desorption buffer. If the buffer contains material that cannot be removed at the peptide level and will interfere with mass spectrometric analysis, the sample must be cleaned by spin filtration, dialysis or by chemical means.
 - 5) Reduction of volume - the sample volume must be reduced to a volume of 1 mL or less for a global digestion. This can be achieved through spin filtration or

lyophilization dependant upon the desorption buffer (this also serves as clean-up to remove possible contaminants added for desorption). However, under testing conditions, this volume can be maintained at a reasonable constant.

- 6) Digestion - the protein in the supernatant from the lysis and spin procedures can then be reduced, denatured, tryptically digested and cleaned up by solid phase extraction for mass spectrometric analysis.

All of these steps must be considered for the removal of protein from microbial communities existing in a natural state on sediment. However for experimentation, the system must remain controlled and modified on the lysis level. Instead of microbial community lysis on the sediment, a known quantity of protein will be added to equally weighed portions of sediment to quantify exact removal rates from each sediment treatment. This is achieved by weighing equal amounts of thoroughly homogenized sediment and adding equal amounts of lysed *Escherichia coli* proteins to each treated and untreated sediment aliquot. This is followed by solubilization of the proteins, collection, digestion and protein identification from each.

Chapter 3

Research Parameters

3.1 Introduction

Background sediment samples were collected at different locations within the *in-situ* test plot on the ground of the former uranium ore processing facility in Rifle, Colorado. The residual groundwater is contaminated with uranium and lies within a heterogeneous alluvial deposit on the floodplain of the Colorado River. The test sediment was collected at a background location which is composed of mostly unconsolidated clay, silt, sand, gravels and cobbles of which 2.4 to 4 m are weathered claystone of the impermeable layer of the Wasatch formation at a depth of 6.1m (Anderson, Vrionis et al. 2003).

3.2 Hydrometer test

Sediment textural analysis was done using a standard hydrometer test. Briefly, the sediment was dried at 80°C overnight and the large particles were sieved out. Sediment (100g) was weighed and added to a Waring blender with deionized (DI) water and a detergent dispersing agent. The sediment was blended for 5 minutes on high and then dispensed into to a 1 L graduated cylinder. The blender was rinsed with water and the resulting solution was added to the cylinder, which was then brought up to 1 L with DI water. The cylinder was inverted and the sediment suspended for 1 minute and the previously calibrated hydrometer was placed in the solution. A reading was taken after 40 seconds to determine the silt and clay content. This was repeated 4 times and the average was used. The reading was then taken after 2 hours to determine the amount of clay. The

readings were corrected according to temperature and the results were analyzed against the U.S. textural triangle.

3.2.1 Hydrometer test results

Old Rifle, Colorado background sediment was determined to be a sandy loam as per the U.S. textural triangle with 70% sand, 5% clay and 25% silt (Fig. 4.1).

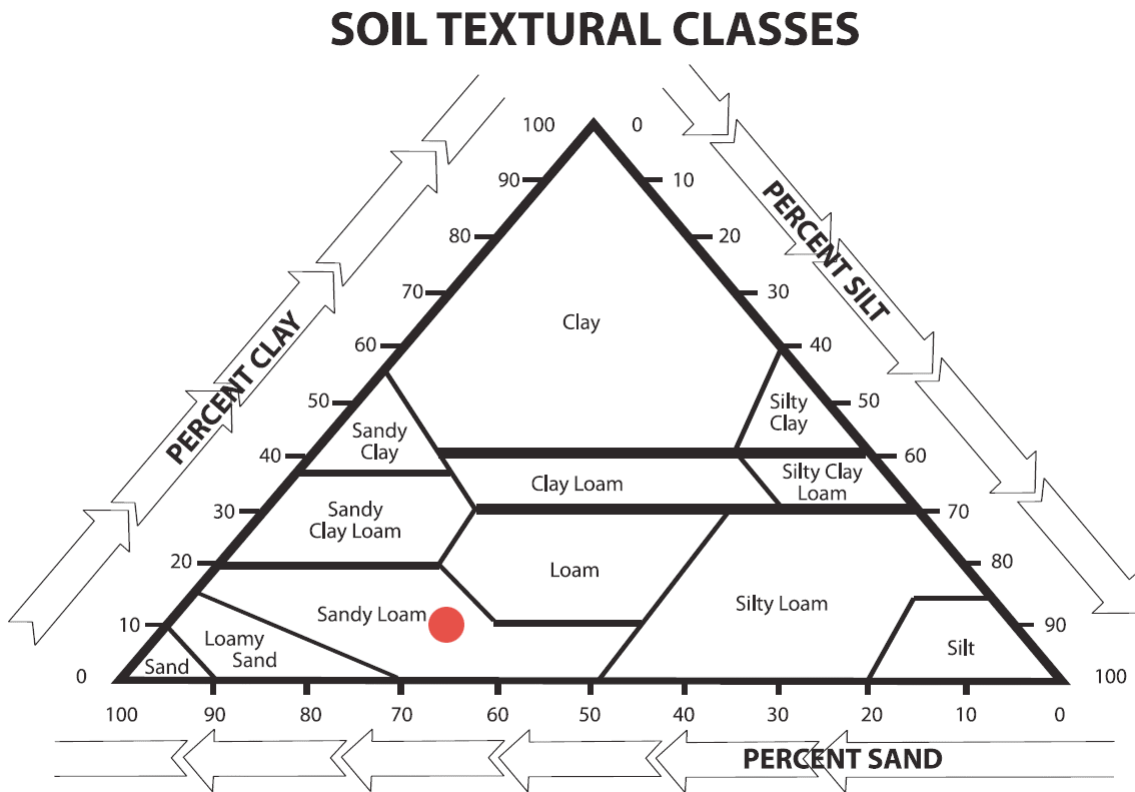


Figure 3.1 - U.S. Textural Triangle (USDA) Classification of Old Rifle, CO background sediment

3.3 Mineralogy

Mineralogy of the whole rock and clay-size fractions was determined by x-ray diffraction (XRD) techniques performed at the Pacific Northwest National Laboratory.

“Bulk samples were prepared by crushing one gram of homogenized samples in an agate

mortar and pestle before packing into aluminum sample holders. Each specimen was analyzed using a Scintag XRD unit equipped with a Pelter thermoelectrically cooled detector and a copper x-ray tube. Individual scans were obtained from 2 to 65° 2 θ (0.01° step) with a dwell time of 2 seconds. Scans were collected electronically and processed using the JADE[®] XRD pattern processing software. Identification of the mineral phases was based on mineral powder diffraction files (PDF[™]) published by the Joint Committee on Powder Diffraction Standards (JCPDS) International Center for Diffraction Data (ICDD) (Newtown Square, Pennsylvania). Semi quantification of mineral phases in the whole rock sediment samples were determined by the whole pattern fitting technique provided by JADE[®] XRD pattern processing software. The software allows whole pattern fitting of the observed data and Reitveld refinement of crystal structures. A diffraction model is fit by non-linear least-square optimization in which certain parameters are varied to improve the fit between the two patterns. Success of the refinement process is measured by a ratio of the weighted and calculated errors. This value, referred to as “goodness of fit”, is expected to be close to one in an ideal refinement. Preparation of the clay fraction for XRD analysis began by dispersing the whole rock sediment. Approximately 100g of sediment was transferred into a 1.0 L bottle and mixed with 1.0 L of 0.001 M sodium hexametaphosphate. The suspensions were allowed to shake overnight to ensure complete dispersion. The sand fraction was separated from the dispersed sample by wet sieving through a # 230 sieve. The sand and silt fractions were separated from the clay fractions by using Stoke’s Law settling. The lower limit of the silt fraction was taken at >1.4 μm . Each clay suspension was concentrated to an approximate volume of 30 ml by adding a few drops of 10N MgCl₂ to the dispersed slurry after silt removal.

Concentrations of the clay in the concentrated suspensions were determined by drying known volumes of the suspension and weighing the dried sediment. The density of the slurry was calculated from the volume pipetted and the final weight of dried sediment. Volumes of slurry equaling 250 mg of clay were transferred into centrifuge tubes and saturated with either Mg²⁺ or K⁺ cations. Clay samples were prepared using the Drever (Drever 1973) method and placed onto an aluminum slide for XRD analysis. Due to the tendency of the clay film to curl and peel off the slide, the Mg²⁺ saturated specimens were immediately solvated with a few drops of a 10% solution of ethylene glycol in ethanol and placed into a dessiccator containing excess ethylene glycol for a minimum of 24 hours. Potassium saturated slides were air dried and analyzed, then heated to 550°C for one hour and reanalyzed ” (Qafoku 2009).

3.3.1 Mineralogy results

Characterization of the sediments by XRD revealed the mineralogy of the sediments are dominated by quartz and feldspar (both plagioclase and alkali-feldspar), with lesser amounts of amphiboles and clays, primarily mica (Table 3.1)(Qafoku 2009).

Table 3.1 - Mineralogy of the Old Rifle Background Sediment (Qafoku 2009)

Sample Number	Mineral Phase (wt-%)						Goodness of fit ¹
	Quartz	Amphibole	Plagioclase	K-Spar	Mica	Calcite	
Bkg. A	56	2	20	15	6	2	3.0

¹ Values closest to 1.0 represent an ideal refinement.

< 2 mm (sand+silt+clay) fraction

Sediment location	Mineral Phase (wt-%)				Normalization Factor
	Smectite	Illite	Chlorite	Kaolinite	
Bkg A	36	50	4	9	1.42

< 2 μm (clay) fraction

3.4. *Escherichia coli* for protein binding tests

Escherichia coli (*E. coli*) is a highly characterized gram negative bacterium with a sequenced and highly annotated genome and *E. coli* protein is not likely to be found in natural Rifle sediment. For these reasons, equal quantities of lysed *E. coli* were added to treated and untreated sediment, subsequently removed, digested and analyzed on a mass spectrometer to determine protein adsorption. This information can then be compared to a digestion of *E. coli* alone (not added to sediment) to determine protein recovery from adsorption onto untreated vs. treated sediment.

3.4.1 *E. coli* Growth Conditions

E. coli strain K-12 MG1655 was grown in 2L of LB broth in a baffled Erlenmeyer flask at 37°C for 6.5 hours at 250 rpm. The *E. coli* was grown to stationary phase and harvested (at a final OD₆₀₀ of 1.370) by centrifugation at 4000 rpm for 30 minutes at 21°C. The supernatant was discarded and the pellets were frozen and stored at -80°C.

3.4.2 Tryptic digestion of *E. coli* samples

The buffer 100mM NH₄HCO₃, pH 8.4 was added to the harvested *E. coli* pellets and vortexed. The sample was transferred into PULSE tubes and lysed with a Barocycler NEP2320 (see section 1.5.2.1). The lysed cells were combined into a 15 mL centrifuge tube and stored on ice to inhibit proteolysis. A bicinchoninic acid assay (BCA) (Pierce, Rockford IL) was performed to determine the protein concentration. Four aliquots of 2 mg of protein were added to 2 mL centrifuge tubes. The remaining lysed *E. coli*, along with two 2 mg *E. coli* aliquots were snap frozen in liquid nitrogen and stored at -80°C. The two *E. coli*-only (no sediment) control samples were reduced and denatured by adding urea to a target concentration of 7 M. Thiourea was added to a target

concentration of 2 M and an appropriate volume of a 50 mM solution of dithiothreitol was added to the sample to reach a concentration of 5 mM. The samples were incubated at 60°C for 30 minutes and subsequently diluted 10-fold with 100 mM NH₄HCO₃ to reduce the salt concentration. A sufficient amount of a 1 M solution of CaCl₂ was added to obtain sample concentrations of 1 mM CaCl₂. The samples were digested for 3 hours with trypsin (Promega, Madison WI) at 37°C at a concentration of 1 unit trypsin/50 units protein. After trypsin incubation, the samples were snap frozen and placed in a -80°C freezer overnight. Samples were then cleaned up using a 1 mL/100 mg C18 solid phase extraction (SPE) column using the following steps: Conditioned column with 3 mL of MeOH then rinsed the column with 2 mL acidified water (0.1% TFA). The sample solution was slowly passed through the column then the column containing the sample was washed with 4 mL of 95:5 H₂O:ACN, 0.1% TFA. Collection tubes were placed under the column and while tubes were closed off from vacuum, 1 mL of 80:20 ACN:H₂O, 0.1% TFA was added. The elution buffer flowed slowly through the column until dry. The sample was removed from the SPE vacuum chamber and concentrated in a speed-vac to a volume of 50µL. A sufficient amount of 25 mM NH₄HCO₃ was added to dissolve any particulate matter on the tube sides, and a BCA protein assay was performed on the sample to determine the final concentration. The sample was snap froze in liquid nitrogen and stored at -80°C until needed for analysis.

3.5 Experiments

While hundreds of tests in various combinations can be performed on Rifle sediment to determine efficient sediment binding site blockage for optimized protein removal, three tests were performed as a solid foundation. The experiments included:

- 1) Test1 - the determination of amino acid combinations to block binding sites on sediment.
- 2) Test 2 - the environmental conditions for strongly binding the amino acid to the sediment.
- 3) Test 3 - the determination if the benefits of a desorption buffer will outweigh the chance it will interfere with mass spectrometer analysis and the fact that the sample must then be cleaned with a spin column prior to digestion.

3.6 Mass spectrometer capillary LC-MS analysis

The HPLC system consisted of a custom configuration of 65-mL Isco Model 65D syringe pumps (Isco, Inc., Lincoln, NE), 2-position Valco valves (Valco Instruments Co., Houston, TX), and a PAL autosampler (Leap Technologies, Carrboro, NC), allowing for fully automated sample analysis across four separate HPLC columns maximizing throughput in proteomic analyses (Livesay, Tang et al. 2008). Reversed-phase capillary HPLC columns were manufactured in-house by slurry packing 3- μm Jupiter C₁₈ stationary phase (Phenomenex, Torrence, CA) into a 60-cm length of 360 μm o.d. x 75 μm i.d. fused silica capillary tubing (Polymicro Technologies Inc., Phoenix, AZ) that incorporated a 0.5- μm retaining screen in a 1/16" custom laser-bored 75 μm i.d. union (screen and union - Valco Instruments Co., Houston, TX; laser bore - Lenox Laser, Glen Arm, MD). Mobile phase consisted of 0.2% acetic acid and 0.05% TFA in water (A) and 0.1% TFA in 90% acetonitrile/10% water (B). The mobile phase was degassed by using an in-line Degassex Model DG4400 vacuum degasser (Phenomenex, Torrence, CA). The HPLC system was equilibrated at 10 kpsi with 100% mobile phase A, and then a mobile phase selection valve was switched 50 min after injection, which created a near-

exponential gradient as mobile phase B displaced A in a 2.5 mL active mixer. A 30-cm length of 360 μm o.d. x 15 μm i.d. fused silica tubing was used to split ~ 20 $\mu\text{L}/\text{min}$ of flow before it reached the injection valve (5 μL sample loop). The split flow controlled the gradient speed under conditions of constant pressure operation (10 kpsi). Flow through the capillary HPLC column when equilibrated to 100% mobile phase A was ~ 500 nL/min .

MS analysis was performed using a ThermoFinnigan LTQ ion trap mass spectrometer (Thermo Scientific, San Jose, CA) outfitted with a custom built electrospray ionization (ESI) interface. Electrospray emitters were custom made using 150 μm o.d. x 20 μm i.d. chemically etched fused silica (Kelly, Page et al. 2006). The heated capillary temperature and spray voltage were 200°C and 2.2 kV, respectively. Data was acquired for 100 min, beginning 65 min after sample injection (15 min into gradient). Precursor MS spectra (AGC 3×10^4) were collected from 400-2000 m/z followed by data dependant MS/MS spectra (AGC 1×10^4) of the ten most abundant ions using a collision energy of 35%. A dynamic exclusion time of 60 sec was used to discriminate against previously analyzed ions.

3.7 Experimental Data Analysis

Tandem mass spectra (MS/MS) were searched against the *E. coli* protein annotation from *Genbank* from the National Center for Biotechnology Institute (NCBI), downloaded on April 2, 2008. The searches were performed using SEQUEST with no restriction on enzymatic cleavage and no modifications. Results from each duplicate condition (biological replicate) and duplicate instrument run (technical replicate) were combined, and peptides observed in 1 duplicate but not the other were included in the

analysis. Peptide identifications were considered valid if they had SEQUEST scores that passed those established by Washburn et al. (Washburn, Wolters et al. 2001). Proteins for each condition were reported using a total number of MS/MS spectra as the abundance estimate and were considered acceptable protein identifications if they contained at least 2 unique peptide sequences. Protein identifications and abundance estimates were clustered using K-means clustering available in the Multi-Experiment Viewer (MeV) program (Saeed, Sharov et al. 2003) from The Institute of Genomic Research (TIGR). Hydrophobicity calculations were based on the total protein sequence.

Chapter 4

Methods and Results from Test 1 – Binding Sediment Adsorption Sites with Amino Acids

4.1 Amino acid classes

Amino acid binding site blocking potentials were tested according to their classification as polar positive, polar neutral, nonpolar neutral and polar negative.

Table 4.1 - Standard amino acids and side chain properties at pH 7.0 (Cooper 2004)

Amino Acid	3-Letter	1-Letter	Side chain polarity	Side chain charge (pH 7)
Histidine	His	H	polar	positive
Lysine	Lys	K	polar	positive
Arginine	Arg	R	polar	positive
Asparagine	Asn	N	polar	neutral
Glutamine	Gln	Q	polar	neutral
Serine	Ser	S	polar	neutral
Threonine	Thr	T	polar	neutral
Tyrosine	Tyr	Y	polar	neutral
Alanine	Ala	A	nonpolar	neutral
Cysteine	Cys	C	nonpolar	neutral
Phenylalanine	Phe	F	nonpolar	neutral
Glycine	Gly	G	nonpolar	neutral
Isoleucine	Ile	I	nonpolar	neutral
Leucine	Leu	L	nonpolar	neutral
Methionine	Met	M	nonpolar	neutral
Proline	Pro	P	nonpolar	neutral
Valine	Val	V	nonpolar	neutral
Tryptophan	Trp	W	nonpolar	neutral
Aspartic acid	Asp	D	polar	negative
Glutamic acid	Glu	E	polar	negative

The amino acids were also tested according to the hydrophathy index which represents the hydrophobic or hydrophilic nature of the amino acid side chains. In

addition to individually classified amino acids, a combination of all amino acids were also tested.

Table 4.2 - Increasing hydropathy index of amino acids from hydrophilic (lower numbers) to hydrophobic (higher numbers). (Kyte and Doolittle 1982)

R	K	N	D	Q	E	H	P	Y	W	S	T	G	A	M	C	F	L	V	I
-4.5	-3.9	-3.5	-3.5	-3.5	-3.5	-3.2	-1.6	-1.3	-0.9	-0.8	-0.7	-0.4	1.8	1.9	2.5	2.8	3.8	4.2	4.5

4.2 Amino acid binding test procedure

The sediment was sieved to remove the large rocks and cobbles. Sixteen 5g sediment aliquots were weighed and 10% (w/v) solutions of the aforementioned amino acid combinations were prepared by adding equal weights of each amino acid within a classification and nanopure water was added to make a 10% (w/v) amino acid solution. Each solution was added at its natural pH. Each treatment was prepared in duplicate. Two 5g sediment aliquots were treated with 2 mL 10% (w/v) polar positive amino acids (arginine, lysine and histidine), two 5g sediment aliquots were treated with 2 mL 10% (w/v) polar neutral amino acids (asparagine, glutamine, serine, threonine and tyrosine), two 5g sediment aliquots were treated with 2 mL 10% (w/v) polar negative amino acids (aspartic acid and glutamic acid), two 5g sediment aliquots were treated with 2 mL 10% (w/v) nonpolar neutral amino acids (alanine, cysteine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan and valine), two 5g sediment aliquots were treated with 2 mL 10% (w/v) of the 5 most hydrophilic amino acids (arginine, lysine, asparagine, aspartic acid and glutamine), two 5g sediment aliquots were treated with 2 mL 10% (w/v) of the 5 most hydrophobic amino acids (isoleucine, valine, leucine, phenylalanine and cystine) and two 5g sediment aliquots were treated with 2 mL water as

the untreated control samples. Each sample was thoroughly mixed and vortexed followed by incubation at room temperature on a rocker overnight to dryness.

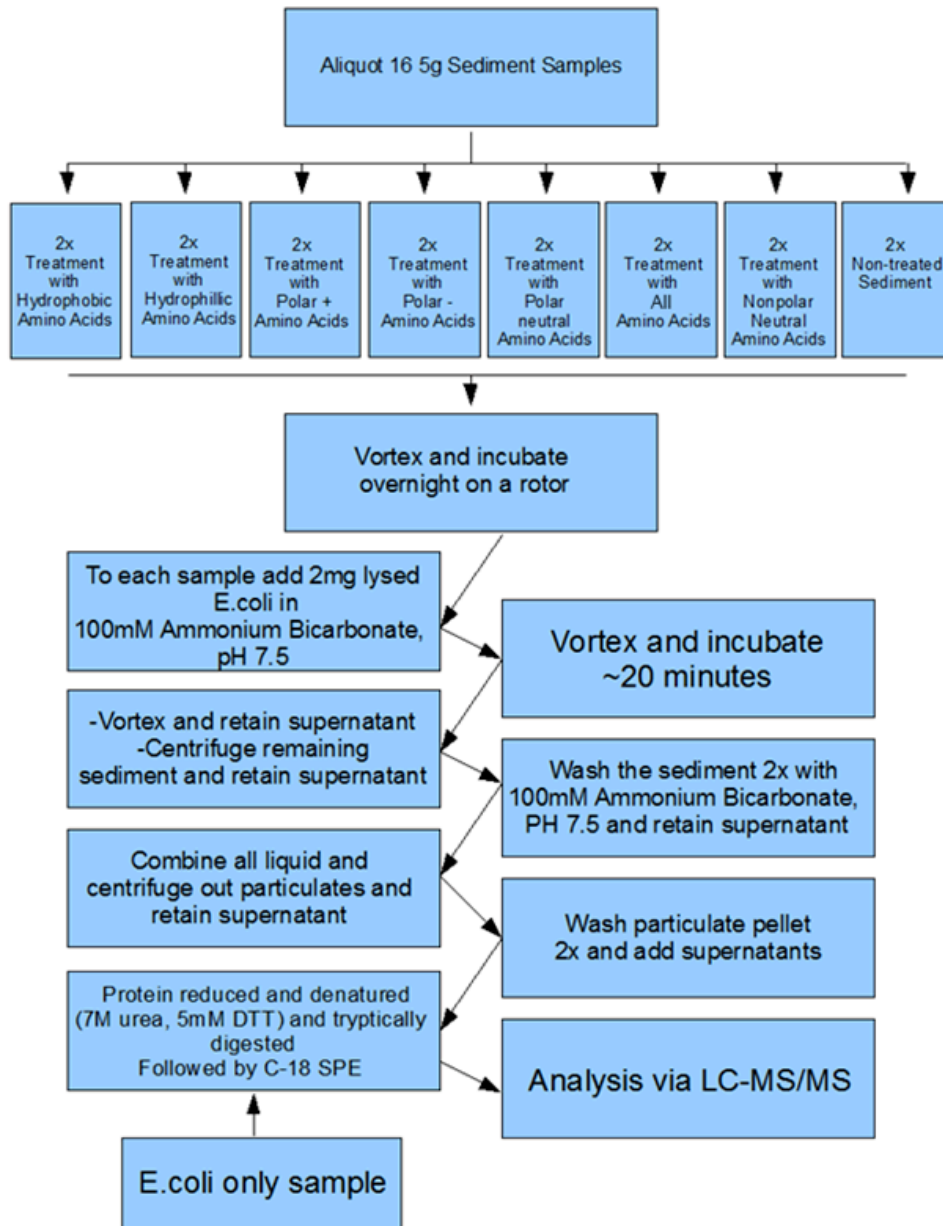


Figure 4.1 - Flowchart of Test 1 - Amino acid binding experimental design

4.3 *E. coli* protein binding

Each treated and untreated sediment sample was reconstituted in 3 mL of nanopure water followed by 2mg of lysed *E. coli*. They were then vortexed and thoroughly mixed followed by 20 minutes of incubation at room temperature on the rocker. The samples were then placed in labeled 50 mL centrifuge tubes and centrifuged at 4000 rpm for 5 minutes. The supernatant was retained and pipetted into fresh labeled 15 mL centrifuge tubes on ice. The sediment was washed with 1 mL 100mM ammonium bicarbonate, pH 8.4, centrifuged again at 4000 rpm for 5 minutes and the supernatant was added to the original samples. Two control *E. coli* samples were digested without addition to sediment. Each sample was digested (see section 3.4.2 – starting with the addition of urea). The samples were then vialled for mass spectrometer analysis.

4.4 Results

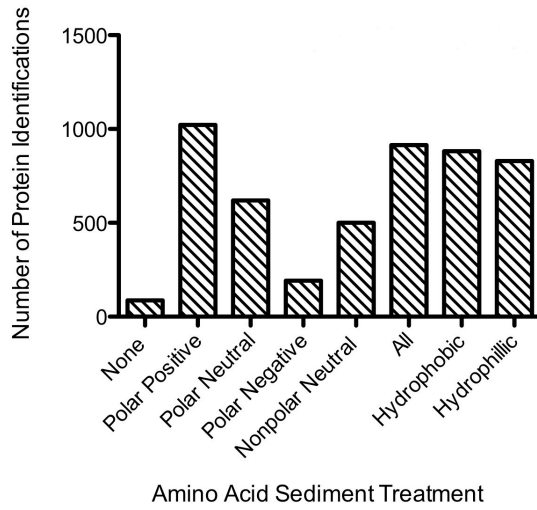
The number of unique peptides and proteins identified from Test 1 are depicted in Table 4.2. These data do not say anything about the reproducibility of the preparations since the peptide identifications from the two biological replicates and the two technical replicates were combined as a pool and the protein identifications were made from the pool (combinational data as opposed to the mean data). As reported in section 3.7, proteins were considered an acceptable protein identification if they contained at least 2 unique peptide sequences. The most peptide and protein combinational identifications compared to the *E. coli* only digestion were observed from the polar positive amino acid treated sediment. Figure 4.2 depicts this data in separate graphs for protein and peptide identifications for comparison purposes. Figure 4.3 depicts the reproducibility of each biological replicate for each amino acid treatment. The peptide identifications from the

two technical replicates per biological replicate were computed combinationally (since technical replicates do not say anything about the reproducibility of the sample preparation so much as the reproducibility of the instrumentation or mass spectrometer runs) and compared to the other biological replicate in order to depict the reproducibility of the sample preparation. The polar positive amino acid treatment had among the best reproducibility.

Table 4.2 – Unique combinational peptides and proteins identified in Test 1

Amino Acid Treatment	Unique Peptides	Unique Proteins
<i>E. coli</i> only	6715	1968
None	293	87
Polar Positive	2659	1022
Polar Neutral	1355	619
Polar Negative	376	192
Nonpolar Neutral	914	500
All	2058	914
Hydrophobic	1784	882
Hydrophillic	2059	829

A)



B)

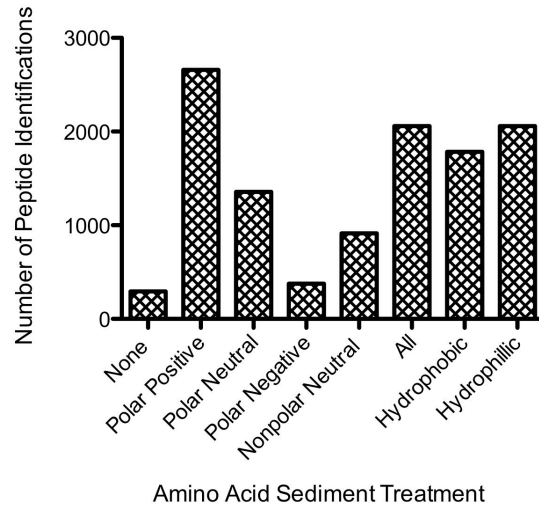


Figure 4.2 – A) Unique combinational proteins identified in Test 1 B) Unique combinational peptides identified in Test 1

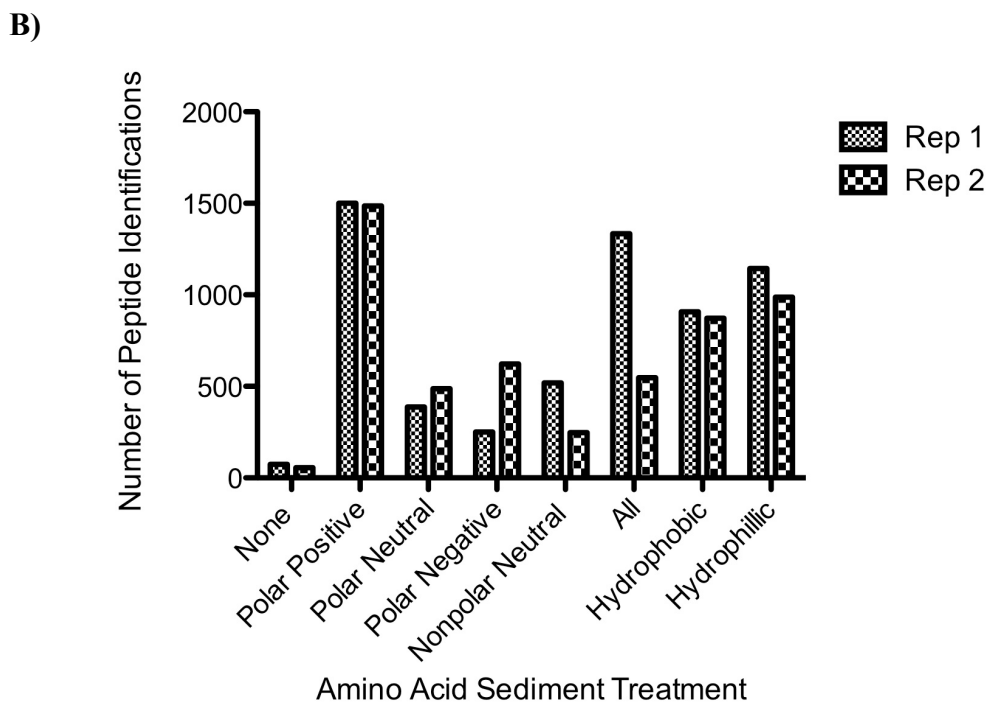
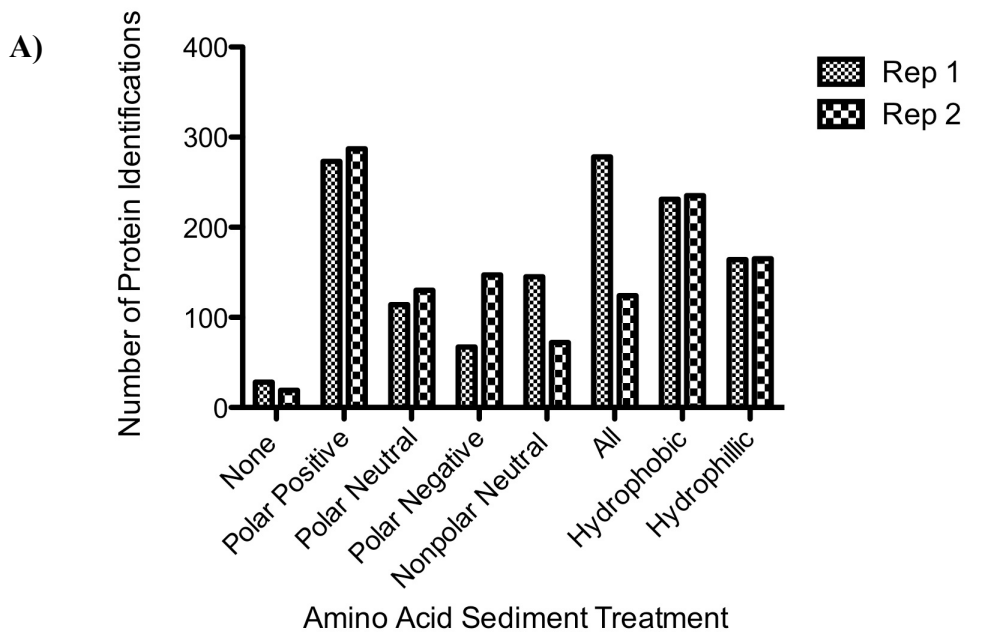


Figure 4.3 – A) Reproducibility of protein identifications from each biological replicate per amino acid treatment in Test 1 B) Reproducibility of peptide identifications from each biological replicate per amino acid treatment in Test 1

Figure 4.3 indicates that the polar positive, hydrophobic and hydrophilic amino acid treatments were the most reproducible. The polar negative, nonpolar neutral and combination of all amino acids had the worst reproducibility. The ranges of the replicates are depicted in Table 4.3 below.

Table 4.3 - Range between biological replicates for each treatment in Test 1

Amino Acid Treatment	Range of Peptide Reps	Range of Protein Reps
None	18	9
Polar Positive	14	14
Polar Neutral	100	16
Polar Negative	372	80
Nonpolar Neutral	272	73
All	785	154
Hydrophobic	35	4
Hydrophilic	156	1

Since the polar positive amino acid treatment resulted in the highest peptide and protein identifications along with the lowest range among replicates, it was compared on a more specific biological scale (protein function) to the untreated sediment results (fig. 4.4).

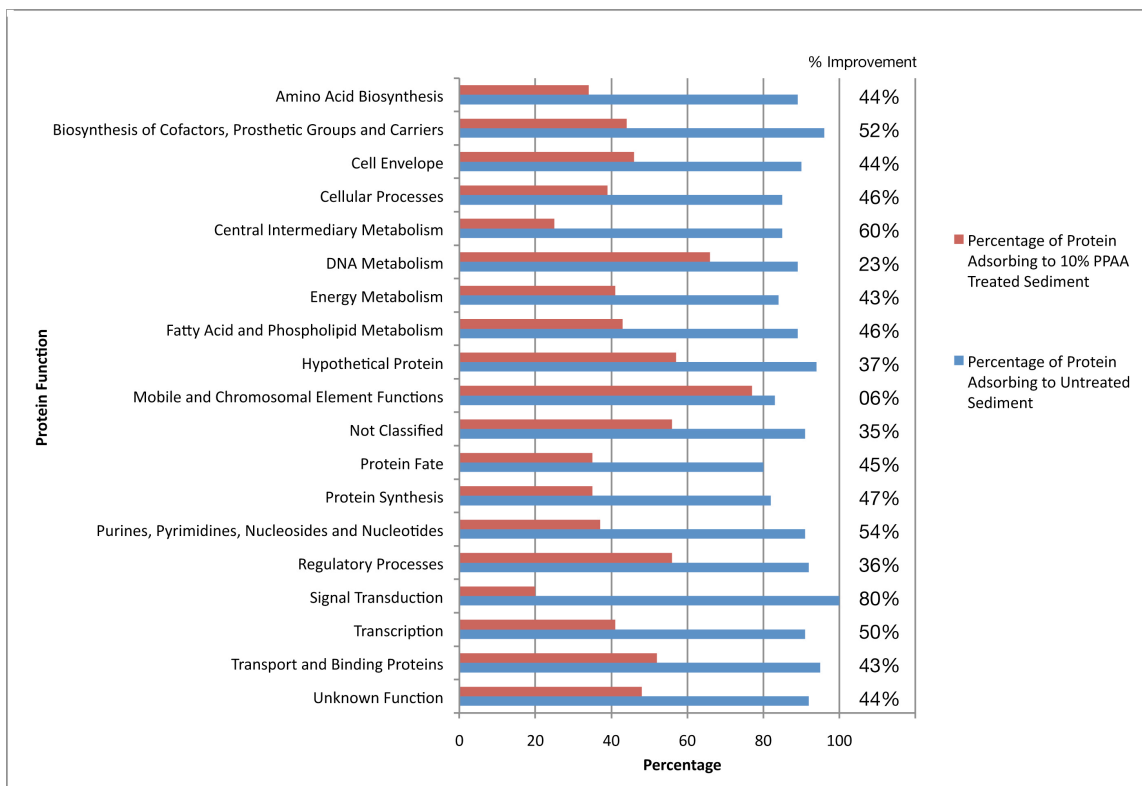


Figure 4.4 - Percentage of protein (categorized by function) adsorbing to 10% PPAA treated sediment (observed in the *E. coli* only digest but not observed in the 10% PPAA treated sample), compared to protein functional categories adsorbing to untreated sediment (observed in the *E. coli* only digest but not observed in the untreated sample). Improvement in reduced adsorption of protein to the 10% PPAA treated sediment over the untreated sediment are reported in percent.

4.5 Discussion

The results from Test 1 clearly show that blocking protein adsorption sites with amino acids increases the recovery of lysed *E. coli* applied directly to sediment. Mass spectral analysis show the number of the *E. coli* peptides recovered from the untreated sediment sample was only 4% of that recovered from the *E. coli* only digestion. These observed peptides resulted in the identification of only 10% of the total identified protein

from the *E. coli* only digestion. This is a clear indication that protein easily adsorbs onto sediment and will not desorb with washing alone. All of the amino acid treatments resulted in an increase in peptide identification from the untreated sample. Also, all of the amino acid treatments (with the exception of the polar negative amino acids) resulted in more protein identification. The polar positive amino acid treatment resulted in the highest identification of peptides and protein with 40% of the peptide identification compared to the *E. coli* only digestion and 52% of the total identified protein. This shows a 36% improvement in peptide identification and a 42% improvement in protein identification from the untreated sediment. Since sediment is negatively charged, the polar positive amino acids are electrostatically attracted and nicely bind to sediment adsorption sites thereby blocking *E. coli* protein from binding to the sediment.

Chapter 5

Methods and Results from Test 2 – Optimization of Binding Conditions

5.1 Test 2 Parameters

The optimization of binding conditions test was used to hone conditions for binding polar positive amino acids to sediment in order to block protein binding sites. Since Test 1 results revealed that the polar positive amino acids (PPAA) performed as the best blocking agent, they were used for Test 2. Conditions tested included temperature, pH, mechanical dispersion, binding time and concentration. Controls included *E. coli* only, *E. coli* only but subjected to the same procedure (to test loss due to the procedure alone and not due to sediment binding) and a standard treatment with parameters including a concentration of 10% polar positive amino acids (PPAA) with a pH 7.0, dispersed in a Waring blender with five one minute cycles with cooling, binding time of overnight at room temperature. Each test followed these parameters except the condition to be tested. A negative control included sediment with no treatment.

The Rifle sediment was sieved to remove the large rocks. Four 5g sediment samples were weighed and set aside for the concentration test. Two 5g sediment samples were weighed and set aside for the no treatment negative control samples. Sediment (200g) was weighed and 100 mL of 10% (w/v) PPAA (3.3g histidine, 3.3g arginine, 3.3g lysine) in water was added to the 200g sediment. Four 7.5g aliquots of the slurry were removed for the “no mechanical dispersion” and sonication dispersion tests. The rest of the sediment was subjected to five one minute cycles of Waring blending on high with cooling between cycles. The slurry was then weighed into 14 aliquots weighing 7.5g each (four samples each for testing pH, temperature and binding time and two samples for

testing the positive control). The 24 sediment aliquots were used for 12 tests with 2 biological replicates each.

5.1.1 Control tests

The two positive control aliquots had the pH adjusted to 7.0 and were placed on a rocker at room temperature for overnight incubation. Two negative control samples were not subjected to the 10% PPAA treatment. Instead the samples had 2.5 mL of water added and then subjected to Waring blending and placed on the rocker at room temperature for overnight incubation.

5.1.2 pH tests

Two samples had the pH adjusted to 4.0 and two samples had the pH adjusted to 10.0 then were placed on the rocker for overnight incubation at room temperature.

5.1.3 Temperature tests

Two samples had the pH adjusted to 7.0 and placed in 4°C fridge with gentle rocking for overnight incubation, while two samples had the pH adjusted to 7.0 and placed in a 50°C oven for overnight incubation.

5.1.4 Mechanical dispersion tests

Two samples that had no blending dispersion had the pH adjusted to 7.0 and placed on the rocker for overnight incubation at room temperature. Two samples with no Waring blending were subjected to 5 cycles of 1 minute probe sonication on ice with 1 minute cooling. The pH was adjusted to 7.0 and the samples were placed on the rocker for overnight incubation at room temperature.

5.1.5 Time tests

Four samples had the pH adjusted to 7.0 and were placed on the rocker at room temperature. Two of the samples were tested after a 2 hour incubation and two of the samples were tested after a 5 hour incubation.

5.1.6 Concentration tests

Two 5g sediment aliquots that were not subjected to the 10% PPAA treatment had 2.5 mL of 1% (w/v) PPAA added and two 5g sediment aliquots had 2.5 mL 50% (w/v) PPAA added and then were subjected to the Waring blender treatment of 5 minutes, pH 7.0 and placed on the rocker overnight at room temperature.

5.1.7 *E. coli* only control tests

Two 2 mg aliquots of *E. coli* only were digested as is, without the addition of any sediment and two 2mg *E. coli* only samples were digested after first being subjected to 20 minutes of incubation at room temperature on the rocker. The samples were then placed in labeled 50 mL centrifuge tubes and centrifuged at 4000 rpm for 5 minutes. The supernatant was retained and pipetted into fresh labeled 15 mL centrifuge tubes on ice followed by tryptic digestion (see section 3.4.2). Following the incubation periods for each sediment treatment, the sample was subjected to the procedure from *E. coli* protein binding section 4.3 followed by the tryptic digestion procedure from section 3.4.2 starting with the addition of urea.

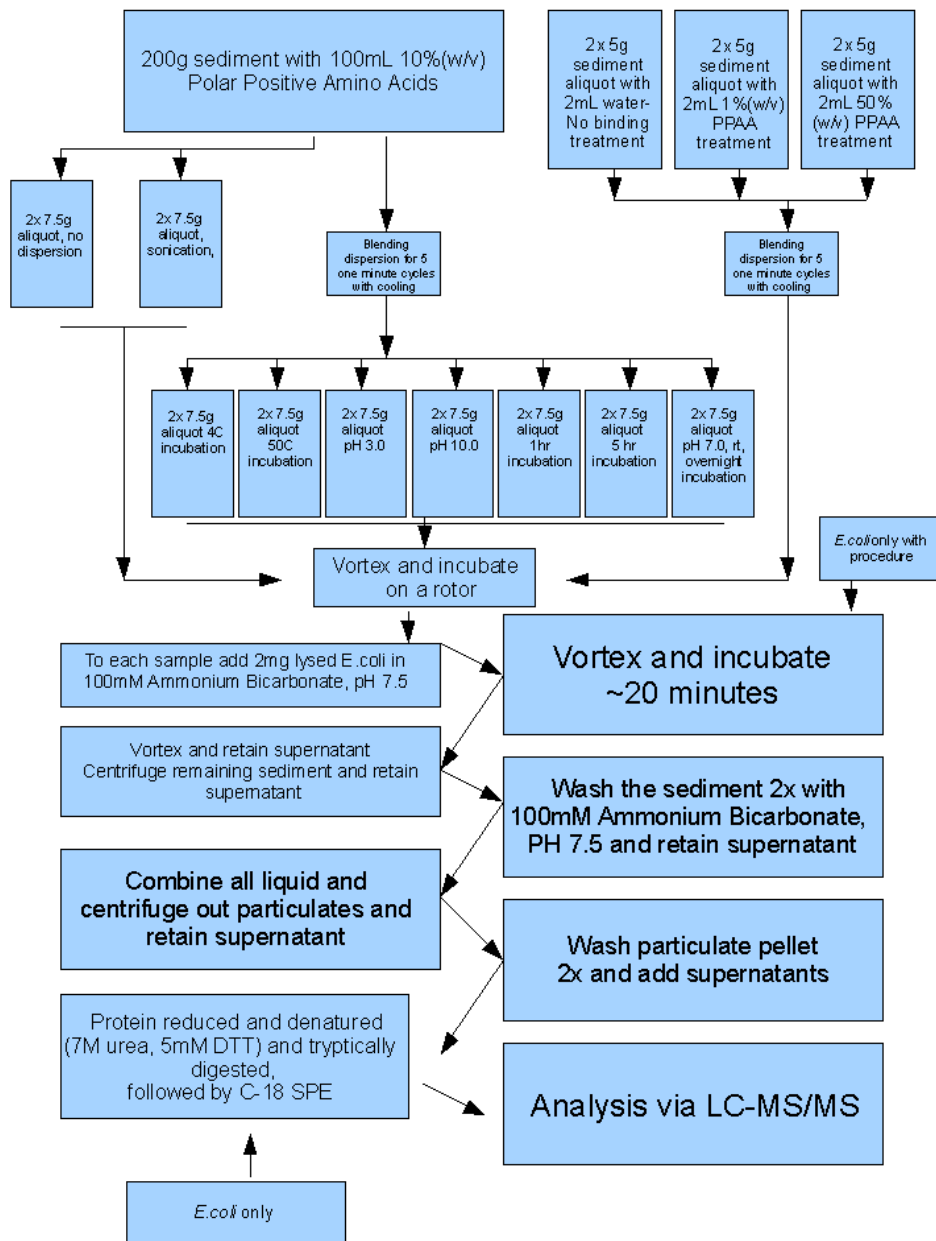


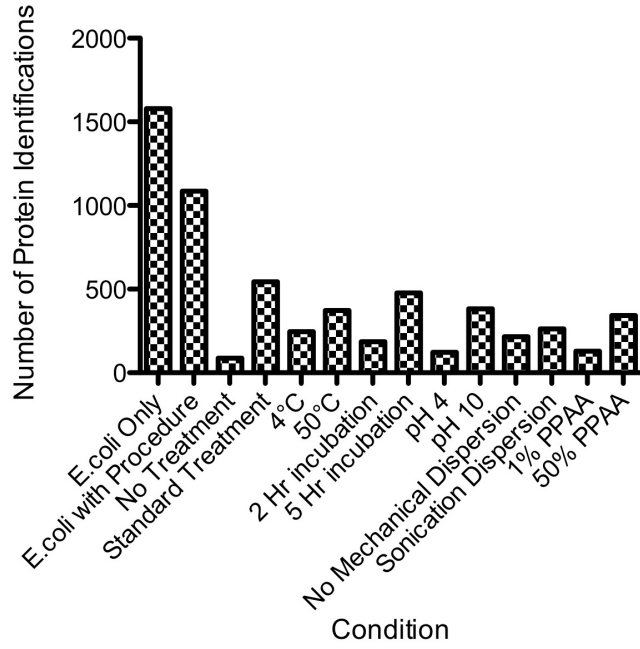
Figure 5.1 - Flowchart of Test 2 - Binding conditions experimental design

5.2 Results

Table 5.1 - Unique combinational peptides and proteins identified in Test 2.

Condition	Unique Peptides	Unique Proteins
<i>E. coli</i> Only (no sediment)	8708	1579
<i>E. coli</i> with Procedure (no sediment)	6511	1085
No Treatment	370	87
Standard Treatment	3218	544
4°C	1477	246
50°C	2464	373
2 Hr incubation	1214	185
5 Hr incubation	2891	477
pH 4	735	123
pH 10	2581	382
No Mechanical Dispersion	1204	215
Sonication Dispersion	1524	262
1% PPAA	518	129
50% PPAA	1989	342

A)



B)

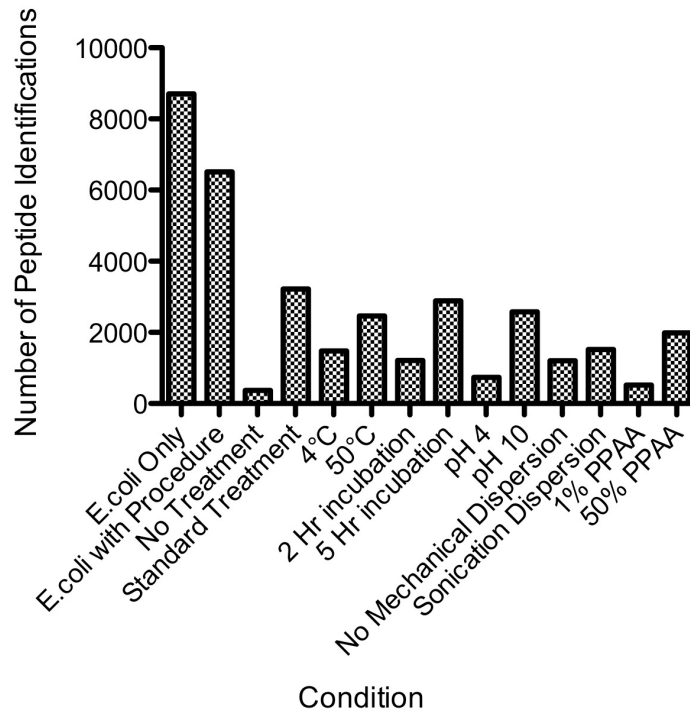


Figure 5.2 – A) Unique combinational proteins identified in Test 2 B) Unique combinational peptides identified in Test 2

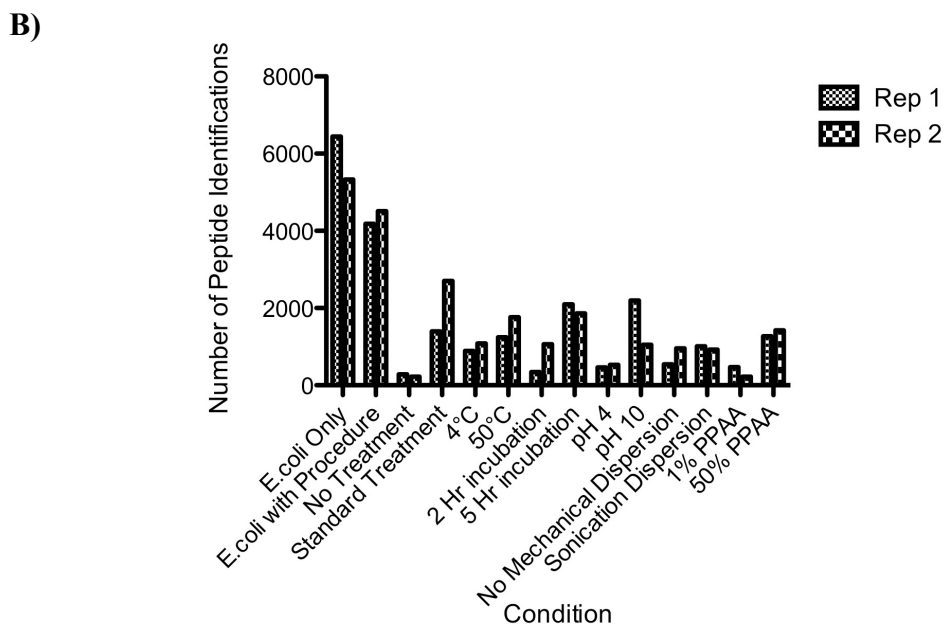
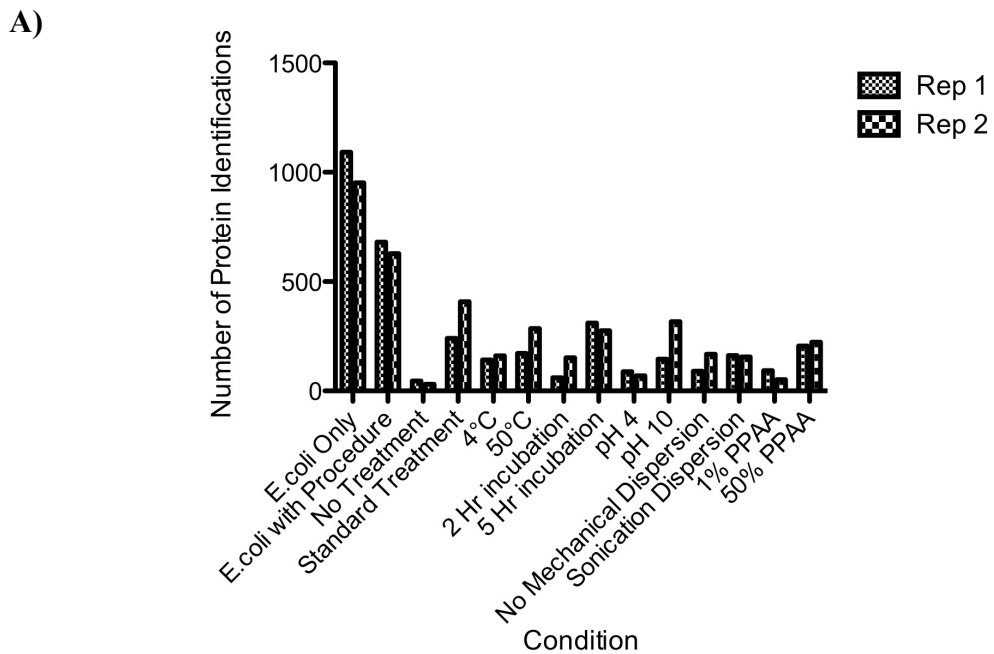


Figure 5.3 – A) Reproducibility of protein identifications from each biological replicate per condition in Test 2 B) Reproducibility of peptide identifications from each biological replicate per condition in Test 2

5.3 Discussion

This *E. coli* digest with the procedure resulted in a 25% decrease in peptide identifications and a 31% decrease in protein identifications from the *E. coli* only digestion. Therefore the sample manipulations needed to process sediment samples result in significant sample losses (fig.4.6).

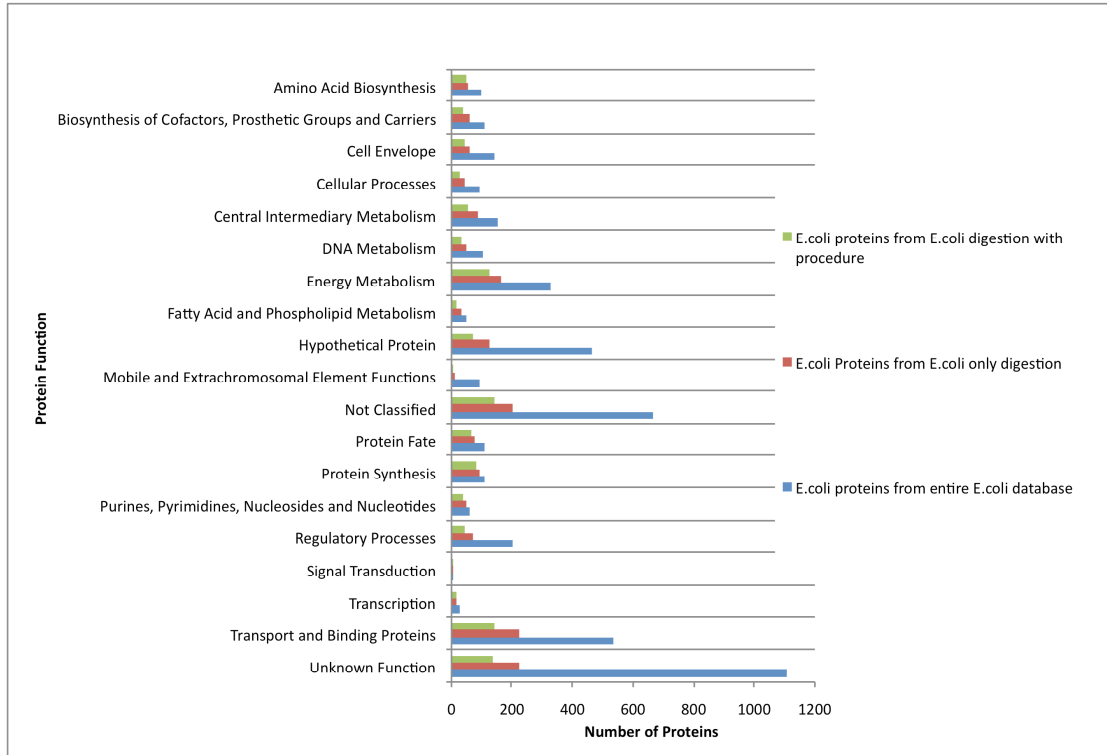


Figure 5.4 – Protein loss by functional category due to sample manipulation and digestion.

Figure 5.4 demonstrates the dramatic protein sample loss observed due to sample manipulation. While over 4000 proteins are expected to be in the *E. coli* proteome, only ~2000 proteins are identified in an *E. coli* only digestion. Not surprisingly, *E. coli* subjected to the same procedure as the sediment samples (but not actually added to sediment) show an even greater loss of protein due to sample manipulation. Considering

the high surface area and physiochemical properties of both protein and sediment, sample loss is expected to be even greater once protein is added to sediment.

The results from Test 2 also indicate that the standard treatment of 10% PPAA at a neutral pH of 7.0 with a room temperature, overnight incubation and Waring blender dispersion resulted in the most peptide and protein identifications. This treatment resulted in 49% of the *E. coli* peptides identified in the *E. coli* only digest with the procedure compared to only 6% of the peptides identified in the untreated sample. This showed a 43% Increase in peptide identifications very similar to the results seen in Test 1. The standard 10% PPAA treatment resulted in 50% of the *E. coli* proteins identified in the *E. coli* only digest with the procedure compared to only 8% of the proteins identified in the untreated sample.

The sediment treatment with 1% PPAA resulted in 84% fewer peptides and 76% fewer proteins identified than the standard 10% PPAA treatment while the 50% PPAA treatment resulted in 38% fewer peptides and 37% fewer protein identifications than the standard treatment. These results indicate that a 1% PPAA treatment is not saturating the sediment particles fully while the 50% PPAA treatment is over saturating the system and causing protein loss.

The Test 2 results indicate that a dispersion step is necessary to enhance complete saturation of binding sites with the PPAA blocking agent. The test with no dispersion resulted in 62% fewer peptide and 60% fewer protein identifications than the standard treatment with a Waring blender dispersion for 5 one-minute bursts with cooling in between. The test with sonication as the dispersion method also resulted in fewer identifications of 53% fewer peptides and 52% fewer proteins than the standard

treatment. This is likely due to the fact that sonication does not induce as much movement of the sediment as the Waring blender treatment and therefore does not achieve as much coverage.

The pH treatment of 4.0 resulted in a very low identification of both peptides and proteins with 77% fewer identifications than the standard treatment of pH 7.0 for both. The pH treatment at the polar positive amino acids natural pH of 10 had better results of 20% fewer peptide and 30% fewer protein identifications than the standard treatment at pH 7.0. As previously hypothesized, lowering the natural pH from 10.0 to 7.0 did increase the identification of both peptides and proteins due to protonation of the ammonium groups on the amino acids making them more positive and thus more likely to electrostatically bond to the mostly negatively charged sediment particles. However, reducing the pH even further to 4.0 gave very poor results likely due to severe acid hydrolysis of the proteins.

Decreasing the incubation temperature from room temperature of 21°C to 4°C resulted in a decrease of peptide identifications by 54% and protein identifications by 55%. This is likely due to the fact that chemical reactions slow at decreased temperature and complete binding reactions did not take place. However, reducing biological and chemical reactions during the incubation period is important to preserve the natural state of the microbial community. Weighing this option is important when deciding on a protocol. Increasing the incubation temperature from 21°C to 50°C also resulted in decreased protein and peptide identifications but with much less loss at 23% peptide identification decrease and 31% protein identification decrease. While binding at the

temperature was most likely fast and strong, it is less likely to have achieved complete coverage during incubation due to quick drying.

Binding agent incubation time showed increase peptide and protein identification with increasing incubation time. Once again the standard treatment of an overnight incubation resulted in the most identifications. The 2-hour incubation resulted in 62% fewer peptide identifications and 66% fewer protein identifications than the overnight treatment. The 5-hour incubation had more identifications than the 2-hour incubation but still resulted in 10% fewer peptide and 12% fewer protein identifications than the overnight treatment.

The Test 2 results indicate that the best sediment binding conditions include 10% PPAA (w/v) concentration at a pH of 7.0, Waring blender dispersion with overnight (18hr) room temperature (21°C) incubation.

In order to account for possible bias of protein adsorption onto sediment by protein characteristic such as pI, mass and hydrophobicity, the entire *E. coli* database, *E. coli* only digestion, *E. coli* digestion with the procedure, the standard treatment with 10% (w/v) PPAA and untreated sediment were plotted to compare trend lines (Figs. 5.5, 5.6, and 5.7).

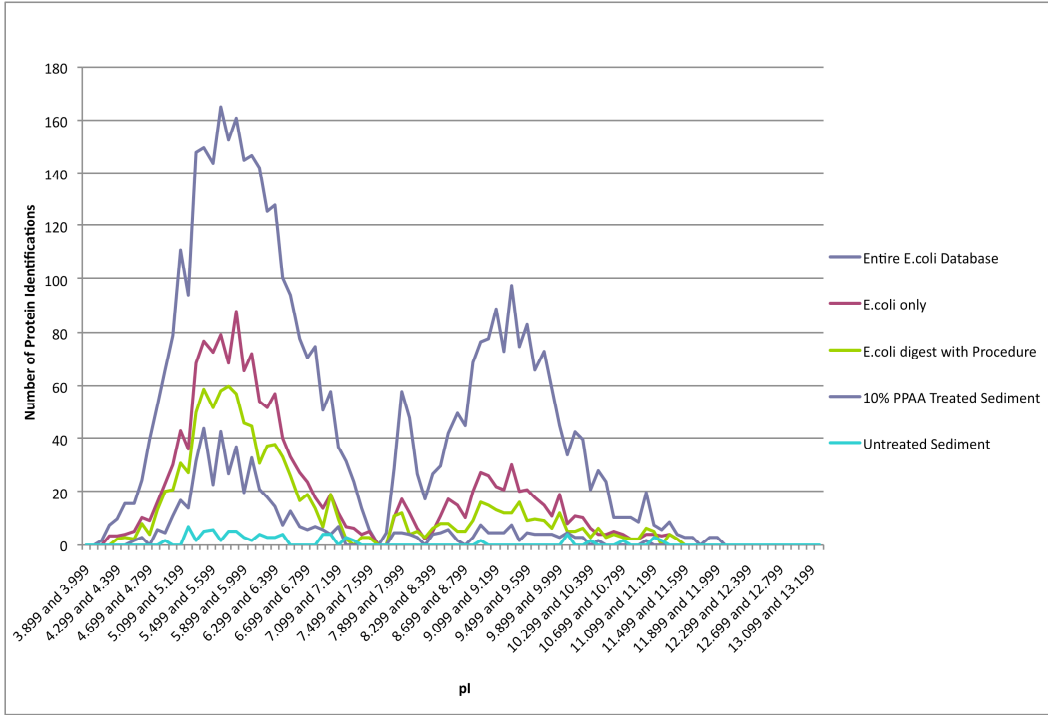


Figure 5.5 - Bimodal pI distribution of combinational *E. coli* proteins identified in Test 2

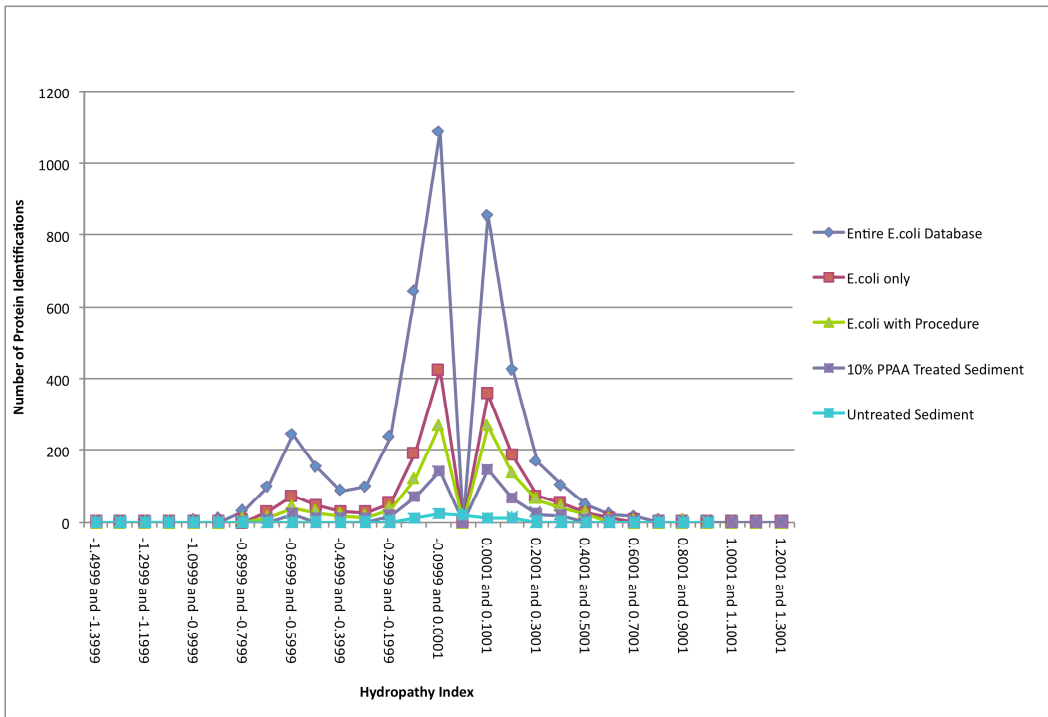


Figure 5.6 - Hydrophobicity distribution of combinational *E. coli* proteins identified in Test 2

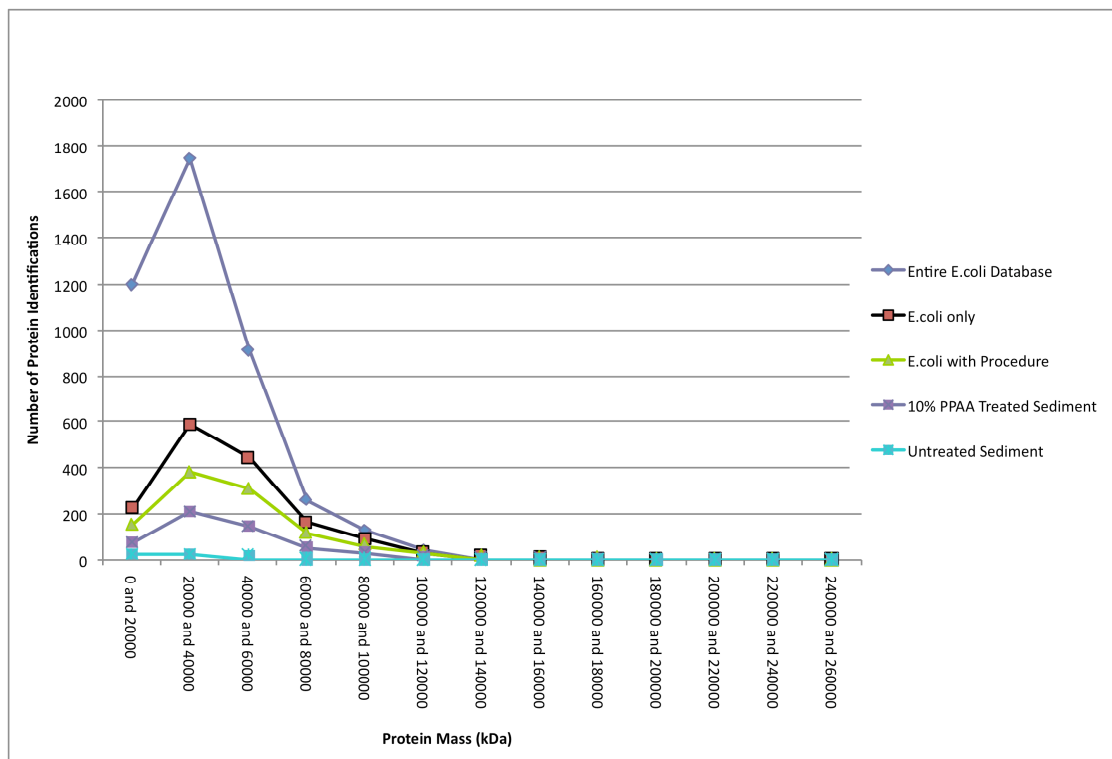


Figure 5.7 - Mass distribution of combinational *E. coli* proteins identified in Test 2

These figures (Figs. 5.5, 5.6 and 5.7) show that protein loss on the 10% PPAA treated sediment, *E. coli* only and *E. coli* with the procedure are consistently distributed regardless of protein characteristic and show the same trend. Therefore, proteins do not show bias towards adsorbing to 10% PPAA treated sediment based on pI, hydrophobicity or mass and seem to be adsorbing in a uniform fashion simply due to increased surface area contact. However, proteins adsorbing onto untreated sediment do not seem to be following the same trend lines and therefore protein adsorption to untreated sediment is much more complex. Clearly, treating sediment with a binding agent such as 10% PPAA not only results in increased peptide and protein identification (reduced protein adsorption) but also reduces the complexity of protein adsorption to sediment.

Chapter 6

Methods and Results from Test 3 - Desorption buffer test

6.1 Test 3 parameters

The desorption buffer test was performed to compare the efficiency of protein removal from 10% PPAA treated sediment with and without a desorption buffer to untreated sediment with and without a desorption buffer. Rifle sediment was sieved to remove large rocks and weighed into eight 5g aliquots. Four sediment samples had 2.5 mL 10% PPAA (w/v) in MES buffer added (pH 5.5). Four sediment samples had 2.5 mL of water added and all samples were incubated at room temperature overnight on a rocker. The following day, two 10% PPAA samples and two untreated sediment samples had 2.5 mL of 100mM NH_4HCO_3 , pH 9.0 added. Two 10% PPAA samples and two untreated sediment samples had 2.5 mL of desorption buffer added. Desorption buffer consisted of 0.5M NaCl, 0.1M raffinose, 0.01% SDS (w/v) and 1mM EDTA in 100mM NH_4HCO_3 , pH 9.0. Lysed *E. coli* (2 mg) was added to each sediment aliquot and incubated for 20 minutes. The samples were then placed in labeled 50 mL centrifuge tubes and centrifuged at 4000 rpm for 5 minutes. The supernatant was retained and pipetted into fresh, labeled 15 mL centrifuge tubes on ice. The sediment was washed with 1 mL of their respective buffers then centrifuged again at 4000 rpm for 5 minutes and the supernatant was added to the original samples. Two control *E. coli* samples were digested without addition to sediment. The samples subjected to desorption buffer were added to Amicon Ultra-15 Spin Filter Units (3K MWCO) (Millipore, Billerica, MA) and cleaned according to the manufacturers instructions. Each sample was digested (see section 3.4.2

starting with the addition of urea). The samples were then vialled for mass spectrometer analysis.

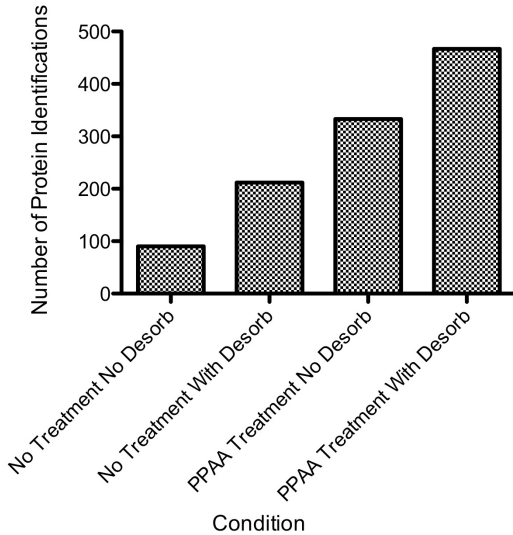
6.2 Results

Unique combinational peptides and proteins detected from Test 3 are depicted in Table 6.1 below.

Table 6.1 – Unique combinational peptides and proteins identified in Test 3

Experiment	Peptides	Proteins
No PPAA Treatment No Desorption Buffer	444	90
Desorption Buffer Only	1072	212
PPAA Treatment Only	2391	333
PPAA Treatment and Desorption Buffer	3816	467

A)



B)

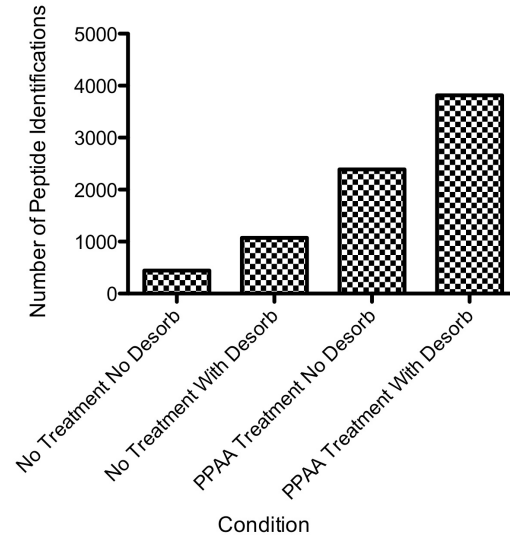


Figure 6.1 – A) Unique combinational proteins identified in Test 3 B) Unique combinational peptides identified in Test 3

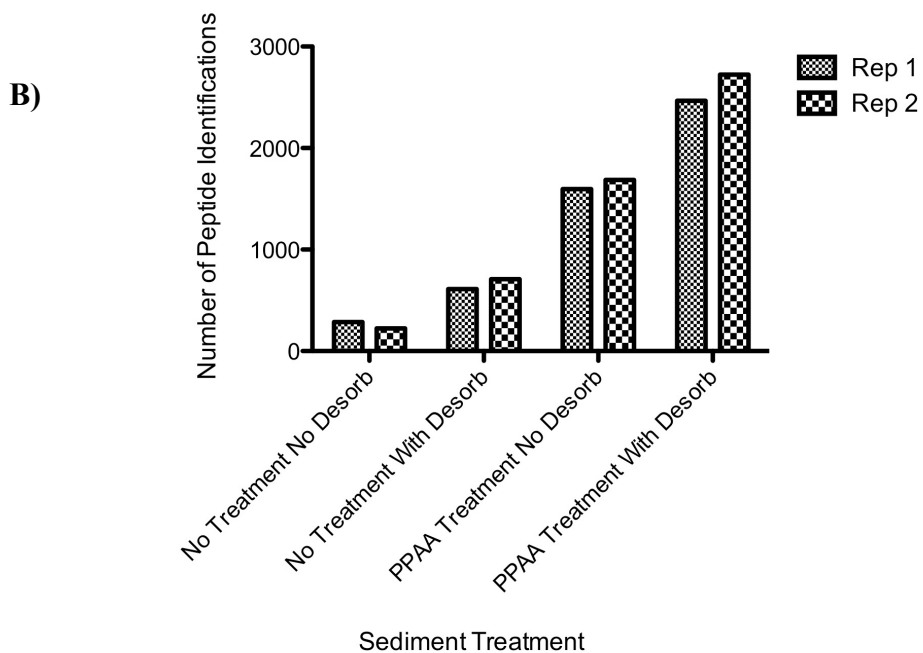
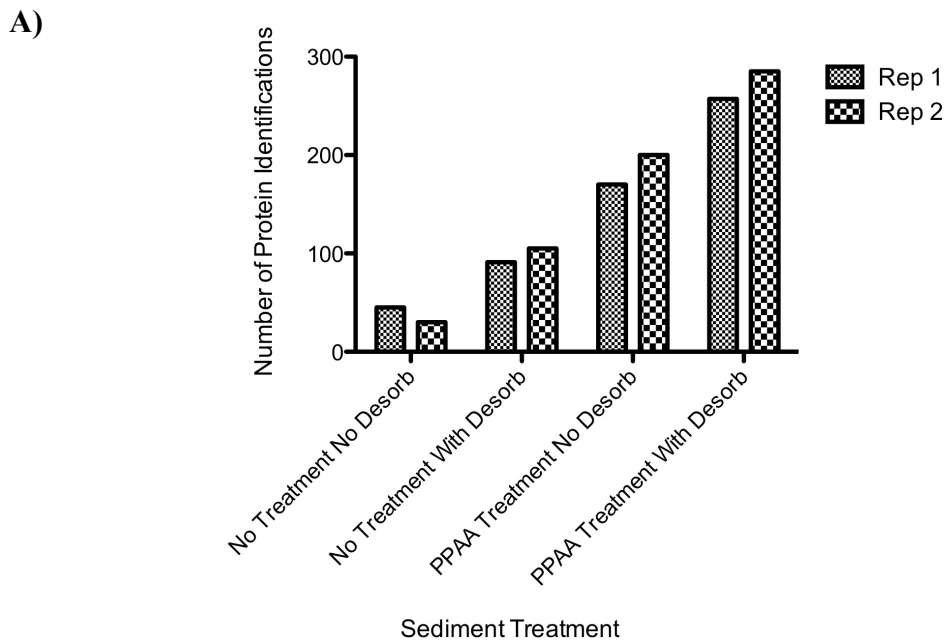


Figure 6.2 – A) Reproducibility of protein identifications from each biological replicate per condition in Test 3 B) Reproducibility of peptide identifications from each biological replicate per condition in Test 3

6.3 Discussion

The results from Test 3 indicate using a desorption buffer alone improves results 2.4 fold from untreated sediment with no desorption buffer. Treating the sediment with PPAA and using no desorption buffer shows a 3.7 fold improvement from the no treatment with no desorption buffer test. Finally, PPAA treated sediment with a desorption buffer shows a 5.2 fold improvement of protein identifications. These results show that a desorption buffer is useful in removing protein from sediment but a significant amount of protein adsorption is taking place (prior to the addition of the buffer) that cannot be released. Treating the sediment with PPAA prior to protein addition with no desorption buffer showed increased protein identifications from the desorption buffer addition alone. This indicates that, while using chemical means to remove adsorbed protein is useful, blocking the binding sites prior to protein addition is more effective in obtaining protein identifications. Combining the use of blocking agents in conjunction with the use of a desorption buffer results in the most protein identifications. While the use of detergents, salts and sugars seem important to solubilize proteins and reduce protein adsorption; they need to be cleaned out of the solution prior to digestion due to interference with trypsin. Therefore, any procedure utilizing a desorption buffer needs to be cleaned with a spin filter which increases protein loss and may counteract any improvement seen by using the buffer. However, these results indicate that even with the spin filter clean-up, a desorption buffer increases protein identification.

The following figures 6.3, 6.4, and 6.5 show the distribution of proteins identified from Test 3 according to the protein characteristics of pI, hydrophobicity and mass.

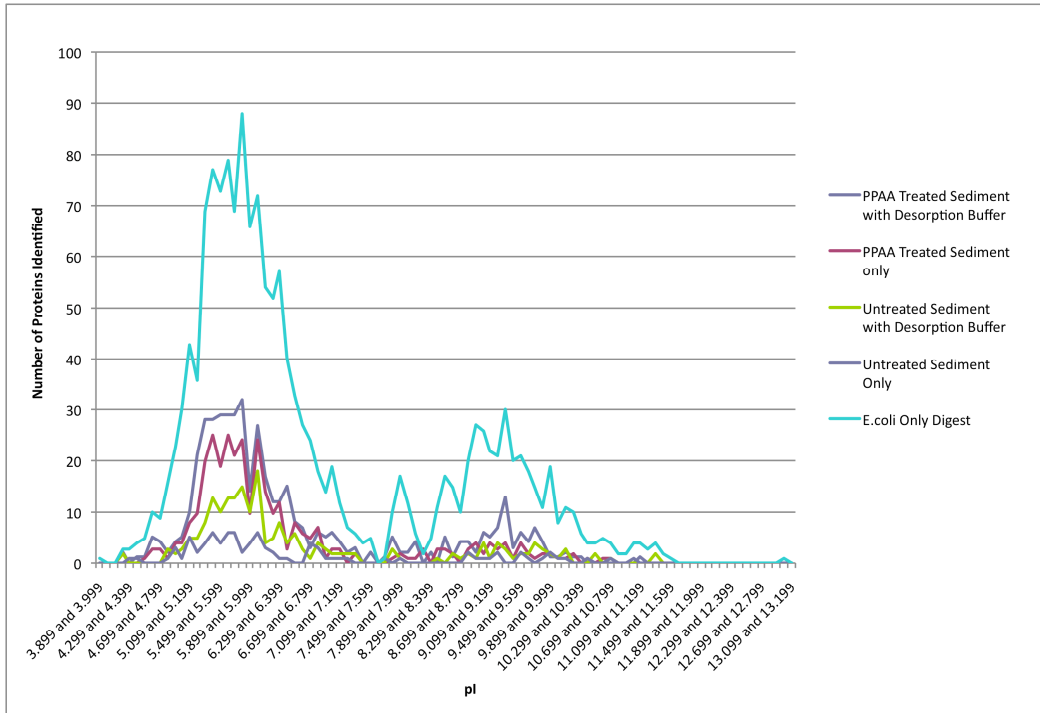


Figure 6.3 - Bimodal pI distribution of combinational *E. coli* proteins identified in

Test 3

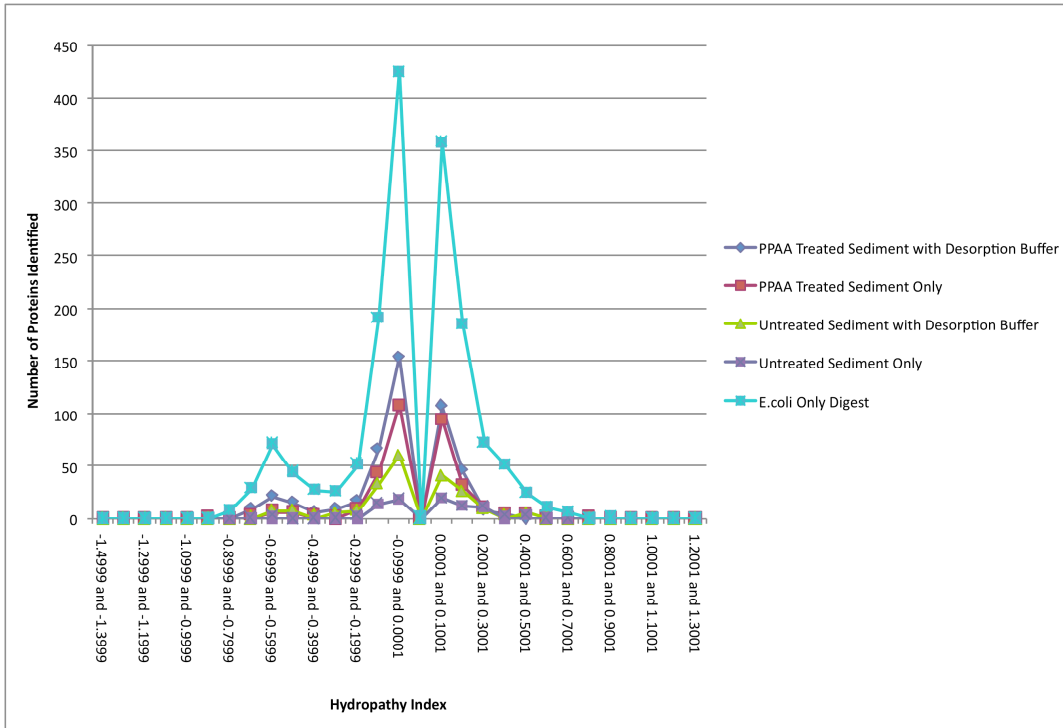


Figure 6.4 - Hydrophobicity distribution of combinational *E. coli* proteins identified in

Test 3

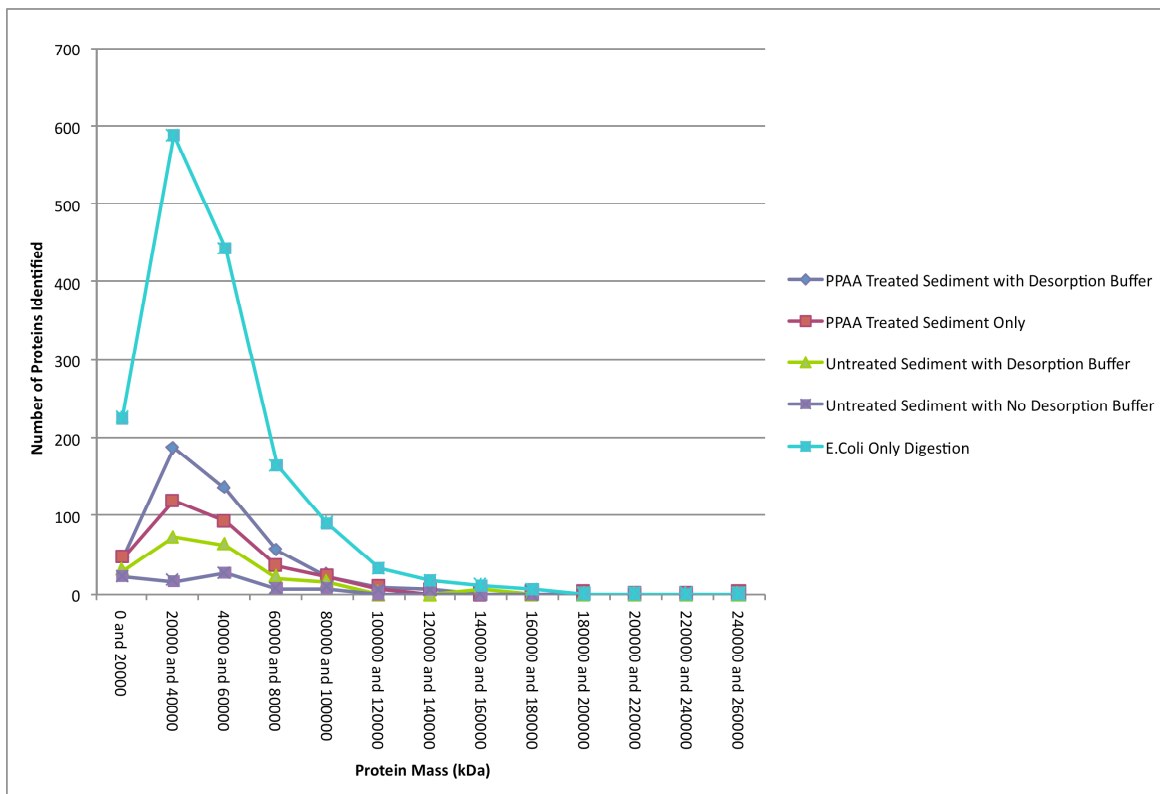


Figure 6.5 - Mass distribution of *E. coli* proteins identified in Test 3

Similar to the results in Test 2, the distributions of proteins according to protein characteristic remain fairly similar regardless of the treatment in Test 3. However, untreated sediment with and without the desorption buffer are both less similar than the PPAA treated sediment. These results once again verify that binding the adsorption sites with a blocking agent reduces the protein adsorption complexity.

Table 6.2 below shows the proteins identified within each treatment and classifies them according to their functional category.

Table 6.2 - Protein functional categories identified per treatment in Test 3. Note that these numbers do not add up to the total number of identified proteins from Table 6.1, because some proteins have more than one function.

Protein Function	No Treatment	Desorb Buffer Only	PPAA Treatment Only	PPAA and Desorb Buffer	<i>E. coli</i> Digest Only
Unknown Function	3	24	48	146	223
Transport and Binding Proteins	4	27	36	133	225
Transcription	5	5	2	11	19
Signal Transduction	1	1	1	3	3
Regulatory Processes	3	10	10	30	68
Purines, Pyrimidines, Nucleosides and Nucleotides	3	6	18	46	47
Protein Synthesis	25	11	12	37	90
Protein Fate	7	10	18	62	77
Not Classified	4	26	35	138	203
Mobile and Extrachromosomal Element Functions	0	3	1	9	13
Hypothetical Protein	1	17	21	77	124
Fatty Acid and Phospholipid Metabolism	1	4	3	14	32
Energy Metabolism	19	25	40	128	165
DNA Metabolism	1	5	7	24	48
Central Intermediary Metabolism	1	11	35	95	85
Cellular Processes	5	6	11	37	44
Cell Envelope	1	9	9	40	61
Biosynthesis of Cofactors, Prosthetic Groups and Carriers	2	8	11	38	60
Amino Acid Biosynthesis	4	9	29	78	56

6.4 Final Conclusions

The results from Test 1 showed that the best amino acid combination for binding protein adsorption sites are the polar positive amino acids (PPAA). Test 2 indicated that the best conditions for binding PPAA are with a Waring blender dispersion at an amino acid concentration of 10% (w/v), a room temperature (21°C) and overnight incubation at a pH of 7.0. Test 3 showed the increased capacity for protein identifications with the use of a desorption buffer (consisting of 0.5M NaCl, 0.1M raffinose, 0.01% (w/v) SDS and 1mM EDTA in 100mM NH₄HCO₃, pH 9.0) in conjunction with the 10% (w/v) PPAA treatment to the sediment.

6.5 Future Directions

The tests included in this study were all conducted on sediment collected from the Colorado River in Rifle, Colorado at a uranium-contaminated site. The composition of sediment is very heterogeneous and varies greatly from one location to the next. Protein adsorption onto sediment varies dependant upon its composition. Therefore, protein desorption tests need to be carried out on various sediment types in order to develop the most appropriate universal protocol or most appropriate protocol per sediment composition. Also, there is a wide array of desorption buffer components and properties that can be further investigated. Many uses of organic solvents, detergents, salts, chaotropic agents, sugars (and many more) can be tested and analyzed with strong emphasis on clean-up and removal of these contaminants for future mass spectrometric analysis. There are also many more binding agents that can be tested and used dependant upon the composition of the sediment.

There are many possible binding agents that can be tested and compared to the use of polar positive amino acids, including casein, non-fat milk, TEPA and spermine.

Once all the factors have been tested and the results finalized, many biological replicates need to be done compared against many untreated replicates in order to more appropriately test the statistical significance of the procedure.

Finally with the use of data obtained from blocking protein binding sites on sediment and the development of an appropriate desorption buffer, actual direct bacterial lysis can be tested with the use of a barocycler to test the efficiency of native bacterial protein from sediment for proteomic analysis and future improvements to bacterial bioremediation of contaminated land.

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