PCR DETECTION OF GROUNDWATER BACTERIA ASSOCIATED WITH COLLOIDAL TRANSPORT

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TABLE OF CONTENTS

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LIST OF TABLES

Abstract

Colloidal transport may increase the amount of contaminant material than that which could be transported by water flow alone. The role of colloids in groundwater contaminant transport is complicated and may involve many different processes, including sorption of elements onto colloidal particles, coagulation/dissolution, adsorption onto solid surfaces, filtration, and migration. Bacteria are known to concentrate minerals and influence the transport of compounds in aqueous environments and may also serve as organic colloids, thereby influencing subsurface transport of radionuclides and other contaminants. The initial phase of the project consisted of assembling a list of bacteria capable of sequestering or facilitating mineral transport. The development and optimization of the PCR amplification assay for the detection of the organisms of interest, and the examination of regional groundwaters for those organisms, are presented for subsequent research.

Introduction

Colloid-facilitated transport of contaminants may be important in cases where contaminants associate strongly with particles such as metals, radionuclides, and hydrophobic organic compounds. In addition, facilitated transport may also be of importance in porous or fractured aquifers with high flow rates, those with high levels of dissolved organic carbon, and those chemically altered or dominated by sodium [24].

Processes involving solute transport have been considered as an equilibrium between the mobile aqueous phase and the immobile solid phase. However, some subsurface environments contain small solid phase particles and macromolecules that can be transported with the mobile aqueous phase [16]. For example, it has been suggested that particles considerably smaller than sand and gravel may promote the migration of compounds in association with the suspended solid phase (in groundwater aquifers) [25].

Colloids are defined as particles ranging in size from 1nm to 1μm that remain suspended for a period of time. Some components of the colloidal phase include SiO_2, illite, muscovite, biotite, calcium silicates (e.g., actinolite), organics, and bacteria [11]. Colloids assume different shapes and are present in various natural systems such as surface, marine, and groundwater environments [27], and colloids in the mobile phase have the potential of sorbing contaminants, thereby causing an increased migration of certain chemicals [16]. However, information on these components and their influence on contaminant transport is limited. Colloidal surfaces can be positively or negatively charged and they can be subjected to both repulsive and attractive forces [22]. The surface area for the smallest colloidal particles is in the order of 10^3 m^2/g [30]. The high surface area per unit mass of colloidal-sized particles provides a significant sorption potential and is of most concern with regard to transport [25,30].

Several radioactive sites have been analyzed for actinides and/or radionuclide migration in groundwater, and in some cases migration has been shown in excess of 100m in groundwater [28]. However, these researchers emphasize the necessity to understand and accurately predict colloid-contaminant migration in groundwater environments.

Goals and Objectives

The goals of this project are to determine if bacteria present in regional groundwaters could serve as colloids capable of transporting radionuclides and to develop a polymerase chain reaction (PCR) assay for their detection. The initial phase of the project consists of assembling a list of bacteria capable of sequestering or facilitating mineral transport. For this, a review of the scientific literature was conducted to determine if bacteria indigenous to subsurface environments are known or could serve as colloids. Subsequent phases of this project include the development and optimization of the PCR amplification assay for the detection of the organisms of interest and the examination of regional groundwaters for those organisms.

Background Information

Colloids

The importance of colloids in facilitating contaminant transport in groundwater systems depends on: the colloid surface area, the amount of reactive sites per unit of surface, preconditioning of surfaces by strongly bound co-sorbates, and the strength of the contaminant-surface reaction [24].

The role of colloids in groundwater contaminant transport is complicated and may involve many different processes, including sorption of elements onto colloidal particles, coagulation/dissolution, adsorption onto solid surfaces, filtration, and migration [27,30]. As a result, colloid transport may increase the amount of contaminant material that could be transported by water flow alone. Negatively charged colloids (e.g., bacteria, organic macromolecules, iron oxides, etc.) are expected to be more mobile than positively charged ones since most groundwater matrices carry a net negative charge [24], thus increasing the migration potential of bacteria capable of serving as colloids for contaminant transport.

Radioelements

Fine particulates produced by bacterial degradation and radionuclides can leach from nuclear waste. Mechanisms of radionuclide migration from a nuclear waste repository include ionic transport, effects of colloids, bacteria, and interactions of ions and ligands [25].

Their migration rate depends on the properties/characteristics of the element, the nature of the aquifer, the solid surface, the presence of colloids, and the presence of natural organic substances (e.g., humic and fulvic acids) [27]. Because radionuclides are more mobile in groundwater than would be predicted based on their adsorption potential, it is likely that some of the material to which they adsorb is mobile [25,30].

Bacteria in Deep Subsurface Environments

Microorganisms are colloidal-size particles [27]. Microbial adsorption and desorption is influenced by the chemistry of the aquifer, the presence of organic compounds (including humic acids), and flow rates. Bacteria can adsorb to solids, aggregate with themselves, and bioflocculate [25]. Bacteria attached to aquifer solids may secrete exocellular materials or release cell fragments to groundwater [24]. Microbial exudates such as metabolites, exocellular polymeric substances, and exopolysaccharides and the microorganism itself serve as important colloids in the subsurface. These exudates may be a source of organic material involved in sequestering metals and other organic substances [15]. Hydrophobic contaminants can sorb to these cell fragments, reducing their hydrophobicity and making them more soluble in water [24]. Geesey and Jang [13] have shown that bacterial polymers exhibit binding affinity for metal cations such as: Cd, Co, Ni, Mn, Zn, Pb, and Cu.

Bacterial cellular activities can remove toxic metals by either reduction to a less toxic form, or binding to cellular structures [4]. Several researchers [17,23,32] have shown bacterial reduction of toxic metals by enzymatic action: $Cr^{VI} \rightarrow Cr^{III}$; $U^{VI} \rightarrow U^{IV}$; and $Hg^{II} \rightarrow Hg^{\circ}$. Harvey and Bouwer [14] mention that bacteria acclimated to contaminated groundwater environments may be involved in co-transport of these contaminants, enhancing their mobility in these environments. It has been stated that the migration or retardation of contaminants depends on the mean transport velocity of the microorganisms, among other factors [29]. In addition, attached subsurface bacteria can become unattached when the nutrient availability increases and/or the concentration of carbon increases, hence augmenting their mobility [24].

Bacterial Migration

Bacterial migration in groundwater is accomplished by diffusion, advection or convective transport, and/or active movement [15]. Microbial transport through aquifers and intact soil cores is faster than chemical tracers because microbial movement is through channels and secondary pore structures rather than entering the intragranular pore space [24]. In a stagnant nutrient medium within a sandstone core, some bacteria can travel 2-4 cm/day [25]. Bacterial movement through soil is influenced by physical-chemical mechanisms, straining, and sedimentation [25]. During their migration, bacteria might lose viability due to starvation, predation, lysis or parasitism. The extent of bacterial sorption might be influenced by the electrostatic charges of both bacterial and solid surfaces, the production of extracellular polysaccharides, and cell hydrophobicity. In addition, microbial migration can be limited by filtration and sorption [12]. It has also been indicated that active mobility of bacteria decreases with decreasing temperatures [29].

Gannon *et al.* [12] suggest a relationship between cell length and migration. In their studies, cells shorter than 1.0μm showed a higher percentage transport than cells $\geq$ 1.0μm. Because they also found that high percentages of cells of larger-sized strains were also mobile in soil, they suggest the influence on migration of other factors in addition to size. Other researchers have shown that transport favors larger bacteria ($>$ 0.6μm) [15].

Bacteria generally have a negative charge regardless of their Gram reaction classification. The cell wall of gram-negative bacteria has an additional outer layer (composed of lipopolysaccharide, phospholipids, and proteins) not present in gram-positive organisms. This outer layer presents a surface with strong negative charge [33]. It may be possible that gram-negative bacteria migrate faster (in negatively charged matrices) than gram-positive organisms of equal size. Both size and Gram reaction classification are parameters to consider when selecting the bacteria for inclusion in this study.

Bacteria Serving as Colloids

McCarthy and Zachara [24] have reported that functional groups on bacterial cell surfaces bind metals. Also, it has been reported that the cell wall of gram-positive bacteria at circumneutral pH contains electropositive amino groups that can react with soluble anions

(e.g., SiO_3^{2-}) through heavy metal cation bridges [36]. From these studies it can be implied that bacteria can indeed serve as colloids capable of transporting radionuclides.

Barton *et al.* [4] compiled a list of bacteria from various sources capable of binding, precipitating, absorbing, depositing, reducing, and transforming various toxic elements. Table 1 presents the names of those organisms followed by its interaction with toxic elements and the mechanism of interaction (when known). In addition to the bacteria listed in Table 1, other researchers have performed studies identifying bacterial genera autochthonous to groundwater environments [7,18]. These genera are: *Acinetobacter, Aerococcus, Alcaligenes, Arthrobacter, Azomonas, Corynebacterium, Moraxella, Paracoccus,* and *Staphylococcus.* These bacteria might also be suitable for radionuclide transport and possible targets of this study.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) amplification is the novel utilization of a chemical sensor method to measure and monitor microorganisms [31]. Assuming 100% efficiency, a succession of repetitive PCR cycles generates an exponential increase of a specific DNA sequence; twenty cycles potentially yield a million-fold amplification of a given sequence. This technique has been used successfully to enhance the detection of microorganisms in matrices such as food [37], soil [35], air [1,2], and water [5,6].

Conventional culture methods may underestimate concentrations because only cells that will grow will be enumerated and identified while non-culturable organisms go undetected. Application of the PCR technique to water sampling permits the amplification of a target DNA sequence, eliminating the requirement for culture or microscopic examination. One advantage of applying this method is an increase in sensitivity over traditional culture methods [19]. Another advantage of the PCR is the rapidity with which results are obtained compared with culture counts [2]. It is possible to obtain results within hours of sample collection using PCR, compared with days or weeks for culture methods. It should be noted that the PCR can be used for the direct detection of any bacteria as long as unique, non cross-hybridizing sequences are available for the specific microorganism to serve as primers for amplification. If there are multiple organisms of interest in a sample,

it may be possible to use multiplex PCR on the same reaction mix to simultaneously amplify several DNA targets of interest [10].

In order to use the PCR technique, sequence information must first be identified for a specific target DNA segment. For amplification to be effective, this segment must be unique to the organism(s) of interest. Once an appropriate DNA sequence has been identified, oligonucleotide primers are selected and synthesized in the laboratory. Following the PCR process, amplified DNA products are usually analyzed by gel electrophoresis owing to its high resolution. In gel electrophoresis the defined length of the amplification product should agree with the expected size according to the sites of the primers when the gel is observed under UV light after ethidium bromide staining. The authenticity of the amplified DNA is confirmed by sequencing, restriction analysis, or nucleic acid hybridization using an oligonucleotide probe complementary to a specific internal DNA sequence within the product. A positive hybridization reaction confirms the identity of the DNA as being from the specific organism of interest. Selected oligonucleotide primers and probes must be tested for sensitivity, specificity, and selectivity.

Targeted Bacteria

Although numerous bacteria are recognized as interacting with toxic elements in the environment (Table 1), little information is known about the DNA sequences that can be used for the selection of oligonucleotide primers. Currently available information on the primers, lysis/purification conditions, amplification protocols and references for some of the bacteria in Table 1 is provided in Tables 2 - 6. The information presented in these tables represents only the initial phase of this study — establishing the groundwork for future studies that will utilize the PCR assay.

Summary

Many investigations have focused on the use of isotopic comparisons and modeling to study radionuclide migration [8]. Because radionuclides are more mobile in groundwater than would be predicted based on their adsorption potential, it is likely that predictions based on modeling may underestimate their actual movement in groundwater [25,30]. The

use of the PCR technique for detection and identification of groundwater bacteria capable of serving as colloids is sensitive and specific. This technique can be considered as an alternative method for groundwater monitoring of specific bacteria. Compared with culture-based methods, PCR is rapid and can be automated, making it possible to perform sample collection, preparation, and analysis of many samples in a few hours. Therefore, the use of the PCR assay for detection of groundwater bacteria capable of serving as colloids will enhance capabilities to monitor contaminant transport flow.

Table 1. Bacteria present in groundwater and their interaction with toxic elements (adapted from Barton *et al.* [4]).

Organism	**Interaction**	**Mechanism of Interaction**
Aeromonas	Se intake	Cytoplasm
Bacillus megaterium	Binds Cd and Sr	Peptidoglycan in cell wall
Citrobacter	Precipitates U, Pb and Cd of insoluble phosphates	Surface enzyme deposition
*Desulfovibrio**	Precipitates heavy metal sulfides	Generation of heavy metal sulfides
Escherichia coli	(1) Binds Sr, Ni, Mn, Pb; (2) Se intake	(1) Outer membrane (2) Cell wall
Flavobacterium	Se intake	Cytoplasm
Micrococcus lysodeikticus	Binds Cd and Sr	Peptidoglycan in cell wall
Pseudomonas aeruginosa	Deposition of U, Ra and Cs within cells	Not known
Pseudomonas maltophilia	Forms electron dense deposits of Pb; transforms Pb^{II} to Pb-containing colloids; and reduces selenate or selenite to Se-containing colloids	Not known
Pseudomonas mesophilica	Transforms Pb^{II} to Pb-containing colloids; and reduces selenate or selenite to Se-containing colloids	Not known
Wolinella succinogenes	Large internal Se granules	Not known

* And related anaerobic bacteria such as: *Archaeoglobus, Desulfobacter, Thermococcus,* and *Pyrococcus* [20].

Table 2. Primer pairs, amplification conditions and references for the genetic amplification of *Aeromonas salmonicida*.

Organism	*Aeromonas salmonicida*
Primers Sequence	5' CTGAAGGCTTGGACCGAAGT 3'
	5' GGTCCGGGTAGTTGTAATCT 3' (plasmid)
	5' CGTTGGATAGGCTCTTCCT 3'
	5' CTCAAAACGGCTGCGTACCA 3' (genomic)
Culture/Lysis	50 mL **lake water sample**
	Centrifuge @ 58,000 x g for 30 min
	Resuspend in 1 mL ultrapure (UP) water
	Centrifuge
	Resuspend in 20 μL UP water
	Place in liquid Nitrogen for 5 min
	Store at -20° C
	For amplification, defrost quickly and use undiluted
	Resuspend one colony (**cultured cells**) in 1 mL UP water
	Dilute 1:100 in UP water
Reaction Mix (V_f = 50 μL; covered with 50 μL mineral oil)	2 μL cell suspension
	50 mM KCl
	10 mM Tris-HCl (pH 8.3)
	1.5 mM $MgCl_2$
	200 μM deoxynucleoside triphosphates
	100 pmol each primer (plasmid or genomic)
	Heat to 98° C x 10 min
	Cool to 80° C
	Add 2.5 U Ampli Taq DNA
Amplification Cycles	5 min @ 95° C
	30 cycles: 1 min @ 95° C, 1 min @ 55° C, and 3 min @ 72° C
	Final extension: 7 min @ 72° C
Reference	[26]

Table 3. Primer pairs, amplification conditions and references for the genetic (plasmid) amplification of *Bacillus megaterium* spores.

Organism	*Bacillus megaterium*
Primers Sequence	5' GGGA(T/C)GA(T/C)GA(A/G)AA(A/G)CA(T/C)TC 3' 3' CC(A/G/T)CG(A/T/C)TG(A/T/C)CA(A/C/G/T)(G/T)C(A/G/T)T A 5'
Annealing Temperatures	50°C, 52°C, and subsequent 26 cycles @ 55°C
Reference	[9,34]

Table 4. Primer pairs, amplification conditions and references for the genetic amplification of *Desulfovibrio vulgaris* and *Archaeoglobus fulgidus*.

Organism	*Desulfovibrio vulgaris* and *Archaeoglobus fulgidus*
Primers Sequence	5' *cctctaga*ATCGG(A/T)ACCTGGAAGGA(C/T)GACATCAA 3' 5' *cctctaga*GGGCACAT(G/C)GTGTAGCAGTTACCGCA 3'
Reaction Mix (V_f = 100 μL)	2 μL [50 pmol/μL] of each primer 5-50 ng DNA 2.5 U Taq DNA polymerase 10 μL [10X] Taq polymerase buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl) 7 μL [1 M] $MgCl_2$ 8 μL [10 mM each] deoxynucleoside triphosphates
Amplification Cycles	30 cycles: 1 min @ 94°C, 1 min @ 60°C, and 1.2 min @ 72°C Final Extension: 9.9 min @ 72°C
Reference	[20]

Table 5. Primer pairs, amplification conditions and references for the genetic amplification of *Escherichia coli*.

Organism	*Escherichia coli*
Primers Sequence	5' TGTTACGTCCTGTAGAAAGCCC 3' 5' AAAACTGCCTGGCACAGCAATT 3'
Lysis/Purification (Product = 100 - 250 μg purified DNA)	Alkaline lysis with 0.5% SDS; [3] Treat with 0.7 M NaCl-1% hexadecyltrimethyl ammonium bromide Chloroform-isoamyl alcohol (24:1) Phenol-chloroform-isoamyl alcohol (24:24:2) extraction DNA precipitation by 2.5 vol of isopropanol Centrifuge @ 12,000 x g for 15 min Wash with cold 70% alcohol Dry under vacuum
Reaction Mix (V_f = 100 μL)	1X PCR reaction buffer (10X PCR reaction buffer contains: 500 mM KCl, 500 mM Tris-chloride (pH 8.9), and 25 mM $MgCl_2$) 200 μM deoxynucleoside triphosphates 0.2-0.5 μM each primer 2.5 U Taq DNA polymerase 1 μg template DNA
Amplification Cycles	3 min @ 95°C 25 cycles: 1 min @ 94°C, and 1 min @ 59°C
Reference	[6]

Table 6. Primer pairs, amplification conditions and references for the genetic amplification of *Pseudomonas aeruginosa*.

Organism	*Pseudomonas aeruginosa*
Primers Sequence	5' GACAACGCCCTCAGCATCACCAGC 3' 5' AGCGCTGGAGCGAATGGGCCAGCG 3'
Culture	Grown overnight in LB broth @ 37°C 1 mL culture washed with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) Resuspend in 100 μL sterile distilled water
Lysis/Purification	5 μL [20%] SDS Incubate @ 60° C x 15 min Purify using a modified Isogene kit (Perkin-Elmer Cetus)
Reaction Mix	38.75 μL sterile water 5 μL [10X] PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, and 0.1% wt/vol gelatin) 4 μL [2.5 mM] deoxynucleoside triphosphates 0.5 μL [100 μM] each primer 1-10 μL template 0.25 μL [0.5 U/μL] Taq DNA polymerase
Amplification Cycles	2 min @ 95°C 25-35 cycles: 1 min @ 94°C, 1 min @ 68°C, and 1 min @ 72°C Final Extension: 7 min @ 72°C
Reference	[21]

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