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Methods for recovery of deep terrestrial subsurface sediments for microbiological studies

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Summary

Methods for the aseptic recovery of sediments from the terrestrial deep subsurface for microbiological analyses are defined. Sediments were recovered from depths >300 m by rotary drilling techniques using bentonite drilling fluids. Four sampling tools were successfully used and compared for their ability to retrieve different types of subsurface materials. Upon retrieval, sediments were pared and processed under anaerobic conditions in a glove bag. Materials were stored under N_2 gas and shipped via overnight express to collaborating investigators. Six quality assurance protocols were incorporated to ensure that appropriate sediments were obtained and to monitor contamination from drilling fluid infringement. Two quality assurance protocols were field-applicable, and four were performed by independent laboratories. The quality assurance protocols provided multiple techniques for detecting 10 mg contamination from drilling fluids $\cdot kg^{-1}$ sediment. These techniques, which proved appropriate for different types of subsurface sediments, provided samples which were deemed acceptable for microbiological analyses.

Key words: Aseptic; Coring; Groundwater; Microbiology; Subsurface sediment

Introduction

Low bacterial numbers observed at depths in soils by early investigators [1, 2] suggested sparse microbial populations in subsurface environments. Although the presence of microorganisms in deep subsurface formations was reported decades ago [3, 4], scientific thought held that microbial activities were restricted to the uppermost centimeters of the earth's crust [5]. Several recent studies have established the presence

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of microorganisms in deep subsurface environments and determined they may influence groundwater chemistry [6–12]. Anaerobic as well as aerobic microorganisms have been isolated [7, 12] and many appear distinctly different from known species or those isolated from surface soils (Balkwill, manuscript submitted).

The majority of subsurface studies have been performed in shallow unconsolidated sands employing split-spoon corers or similar samplers driven into the sediments by hydraulic pounding [6, 10, 11]. Other samples have been obtained from coring devices which allowed drilling fluids to surround the sample during coring [12]. Once cores were retrieved from subsurface sediments, mechanical paring devices were utilized to remove the outermost sediments which were most likely contaminated by drilling fluids [12]. Additionally, sample cores have been broken and subsampled in the radial-center portion [6] or scraped to remove contamination [12].

At depths >100 m, the drilling techniques became more complex to acquire undisturbed samples with rotary coring. Such drilling requirements increased the need for protocols to assure acceptable quality of undisturbed subsurface sediments used for microbiological analyses. Aseptic sampling of the deep terrestrial subsurface is very expensive, is rarely done, and often the samples are not free of surface and ancillary contamination. It is virtually impossible to drill a sterile well. The purpose of this study was to sample in situ microbial communities without contamination by the drilling operations.

Monitoring the contamination of drilling fluids into the sampling activities represents a formidable task in deep subsurface coring programs. Use of biological agents as tracers of fluid movement in subsurface formations has included *Saccharomyces cerevisiae, Serratia marcescens*, and *Bacillus* sp. [13, 14] but regulators are hesitant to approve the mixing of large concentrations of foreign microorganisms into subsurface aquifers. The use of ionic species as conservative tracers of groundwater flow is well established [11, 14] and applicable to drilling fluids. Recent studies have incorporated fluorescent beads approximating the size of bacterial cells [11] and have demonstrated their movement in subsurface formations.

The objective of this report is to describe sampling procedures and quality assurance protocols developed for the Microbiology of the Deep Subsurface Environments Program sponsored by the US Department of Energy. The major scientific goals of the Department of Energy's Program are: (1) to conduct fundamental research on the biological, geochemical, and hydrological processes that control the mobilization and transport of trace metals, organic compounds, and radionuclides in deep subsurface sediments and groundwater; (2) to investigate the presence, abundance, distribution, and diversity of microorganisms in the deep terrestrial subsurface; (3) to investigate the factors controlling microbial presence and activity; (4) to determine the capabilities and functions of indigenous microorganisms and communities, e.g., metabolic activities and rates, biotransformation processes, growth rates, and unique capabilities; (5) to determine the differences between deep- and near-surface microbiota; and (6) to investigate the implications for mitigating contamination through stimulation of the indigenous microorganisms or the introduction of bioengineered strains and the potential environmental impact of these manipulations on subsurface ecosystems. Addressing Department of Energy goals required the development of drilling procedures and quality assurance protocols substantiating that the drilling hardware, drilling

fluids, or processing did not significantly contaminate subsurface sediments used in microbiological investigations.

The reported protocols enabled the aseptic collection of undisturbed samples from subsurface sediments with minimal yet applicable levels of contamination at depths >300 m in a variety of sedimentary formations, ranging from coarse unconsolidated sands to dense compacted clays. Six quality assurance measures were incorporated into the program to determine the level of drilling fluid contamination of the sediment samples. Two measurements were field applicable and performed prior to sediment disbursement. Four other quality assurance measures were performed by independent laboratories. The sampling and quality assurance program enabled multiple quality assurance determinations capable of detecting drilling fluid contamination of 10 $mg \cdot kg^{-1}$ sediment.

Materials and Methods

Site description

The Savannah River Plant (SRP) is a 768-km^2 restricted access area in South Carolina set aside by the US Government in the 1950s for the production of national defense materials. The facility was operated for the Department of Energy by the E. I. duPont de Nemours and Co. until 1 April 1989. Groundwaters in sections of the plant have been affected by effluents from the site's manufacturing and processing facilities [15]. SRP is located ≈32 km southeast of the Fall Line that separates the Piedmont from the coastal Plain, and is located within the Upper Atlantic Coastal Plain on the Aiken Plateau adjacent to the Savannah River (Fig. 1). Unconsolidated sediments extend to

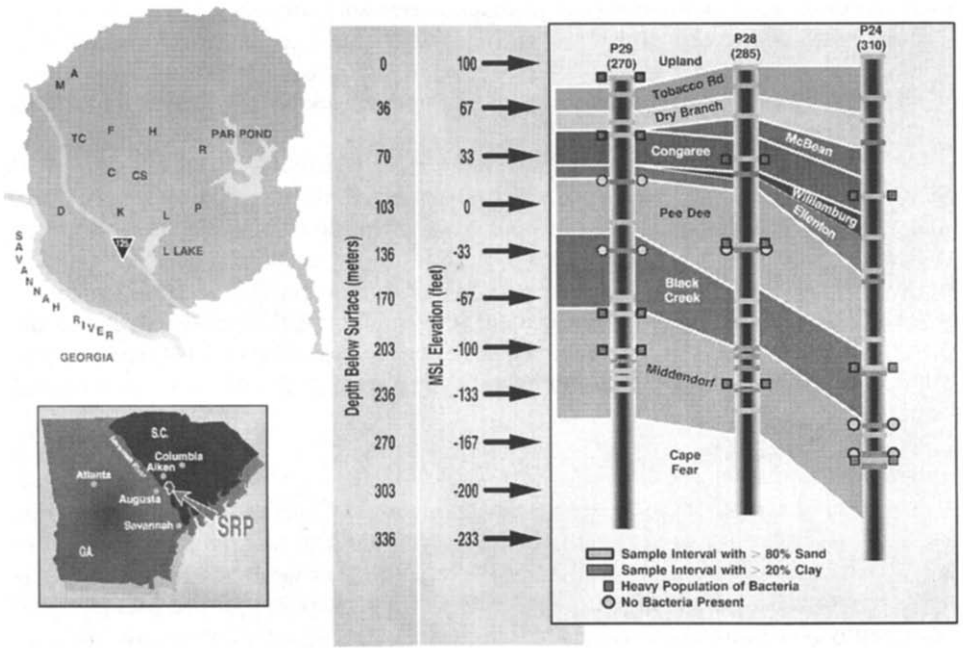

Fig. 1. Map of microbiology boreholes and subsurface formations.

depths of 400 m and are underlain by crystalline metamorphic rock or consolidated mudstone.

Geologic formations beneath the SRP, which were sampled in this study, are shown in Fig. 1 and listed below.

Upland Unit consists of mottled very clayey sand to very sandy clays commonly with pebble zones.

Tobacco Road Formation consists of laminated clayey, silty, poorly sorted, fine to very coarse sands with pebble zones commonly near the base.

McBean Formation consists of very fine to medium sands with calcareous sands, limestones, and marls.

Congaree Formation is a moderately well sorted, fine to very coarse, subangular to subrounded sands with thin gray to green clay layers especially near the middle and base of the formation.

Williamsburg Formation consists of micaceous, silty, very fine to coarse sands to micaceous, sandy, silty clays. The unit is distinguished from the Congaree sands by the presence of mica and a high silt or clay content and from the underlying Ellenton by a light gray or brown to white color.

Ellenton Aquifer consists of silty, lignitic, medium to dark brown or gray sands and gray to black clayey silts and silty clays.

Pee Dee Formation (Upper Middendorf) consists of a light gray, micaceous, clayey, very fine to coarse sand with pebble zones and clay balls to thick layers of variegated clays and silty clays. The sediments are distinguished from the Black Creek Formation by the light color of the sands and variegated color of the clays.

Black Creek Formation (Upper Middendorf) consists of silty, lignitic, medium to dark brown or gray sands and gray to black clayey silty and silty clays.

Middendorf Formation consists of gray to brown, micaceous, clayey or silty, fine to coarse sands with occasional pebble zones grading upwards into thin to thick silts or silty clays. Wood fragments and iron sulfide nodules are occasionally found in this formation.

Three drill sites were sampled in this study: P29, P28, and P24 (Fig. 1). Site P29 was closer to the area where groundwater is thought to recharge the deeper aquifers than the other two sites and generally contained fewer and thinner clay beds. Site P24 was further from the recharge area and each formation was thicker more defined and the subsurface contained more confining clay layers. The marine calcareous deposits of the McBean Formation were most evident at P24 including the recovery of shark-like teeth from recirculated drilling fluids. Because the location of P24 was further downgradient from the recharge area, the drilling penetrated >300 m of unconsolidated sediments.

Sample procurement

Professional Service Industries (PSI) (Jackson, South Carolina) were contracted for the drilling. Two borings pertinent to the microbiological sampling program were drilled at each site. Prior to the drilling of the microbiology hole for undisturbed samples, a continuously cored boring was drilled using a wireline tool to ascertain the stratigraphy and lithology at each site, familiarize the sampling crews with the sediments, and determine the exact locations for the undisturbed microbiological samples

in the second hole. Sample sites were chosen so that adequate materials could be obtained from each major lithologic unit. Consequently, only those regions consisting of similar sediments ≥ 4 m thick were considered for sampling.

Collection of sediment samples from the undisturbed microbiological hole, located $\approx$ 10 m from the exploratory boring, commenced within 4 days of the completion of the exploratory boring. Drill crews, along with onsite geologists, worked 12-h shifts to collect the samples as quickly as possible. Knowledge of the sediment stratigraphy and lithology permitted the core hole to be drilled with a rotary bit between undisturbed sampling depths and continuously flushed with recirculated sodium bentonite viscosifying drilling fluid (Quik-gel, NL Baroid/NL Industries, Houston, Texas). Recirculated drilling fluid was passed through a settling basin and into a holding basin where the viscosity was readjusted. Prior to sampling for microbiological analyses, the entire drill string was removed from the core hole and the drill bit replaced with the sampler appropriate for the sediment to be sampled. The drill string was then replaced into the hole and the sample cored by rotary drilling.

There were three major sources for possible contamination of microbiological samples; drilling hardware, drilling fluids, and processing samples. Various sampling tools (Fig. 2) were employed to retrieve undisturbed aseptic materials for microbiological investigations. The important common features among samplers were that drilling fluid did not circulate through the core liner and all down-hole tools, including drill rods and drill bits, were steam-cleaned or autoclaved prior to use. These features reduced contamination by the drilling hardware and drilling fluids. A Dennison corer (Fig. 2a) (Acker, Scranton, Pennsylvania) utilized a stainless-steel basket as a core catcher to retain unconsolidated sands and housed a brass core liner (6 $\times$ 60 cm) inside of a steel case. A boot extended $\approx$ 2 cm beyond the tip of the case to core sediments without contact with the drilling fluids. Because of the small sample size, the shortness of the boot, and the use of a retaining basket, the Dennison corer resulted in the greatest drilling fluid contamination and consequently was used as few times as possible.

Shelby tube samplers (Acker) (Fig. 2b) were well suited for recovering soft clays. A stainless-steel core liner (7.5 $\times$ 76 cm) was attached to a Shelby tube adapter and lowered into the core hole. Caution was required to ensure that recovered materials matched the lithology of the sample zone rather than being sediments scraped from the side of the core hole. Because no sampling boot was involved, the core tube cut into clay sediments at the bottom of the hole resulting in clean recoveries of sediments. In dense clays, core liners had a tendency to collapse, bend, or break. Breaking of Shelby tubes was more than an inconvenience since it took hours to retrieve a bent sampler from the core hole and repeat the sampling.

A Pitcher barrel (Pitcher Drilling Co., Palo Alto, California) (Fig. 2c) was used to retrieve silty or clayey sediments. A stainless-steel core liner (7.5 $\times$ 76 cm), as used in the Shelby sampler, was placed inside a steel case attached to an outer drill bit. The case contained a spring that pushed the core liner in front of the drill bit and into the bottom sediments. Pitcher barrels were useful in recovering dense clay and silt layers which collapsed the Shelby core liners.

A Phosphate barrel (PSI) (Fig. 2d) provided excellent recovery of consolidated sediments. The sampler consisted of a double mud valve that allowed drilling fluids to pass freely through the sampler during its travel down the borehole. At the bottom

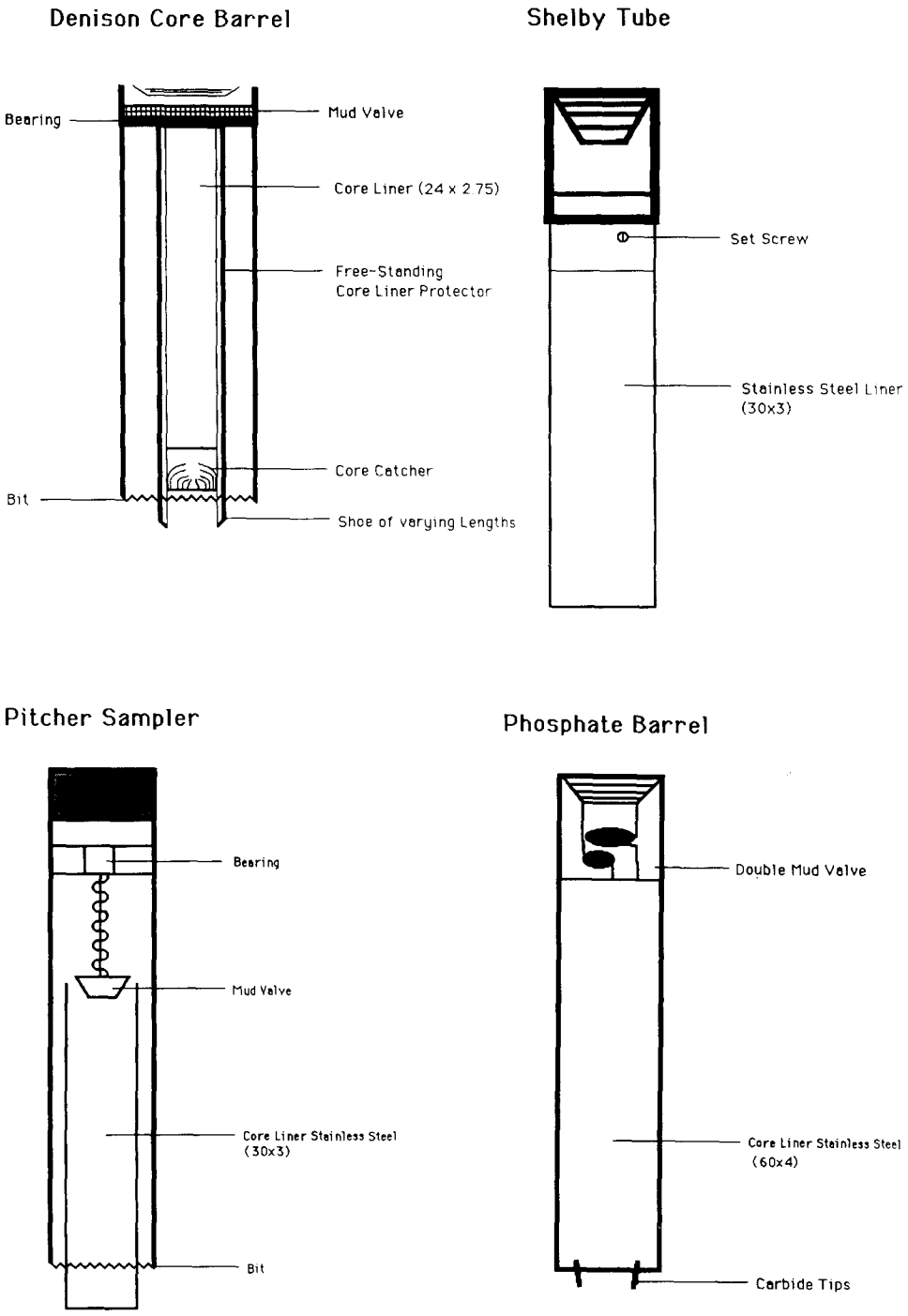

Fig. 2. Sampling tools used for retrieval of subsurface materials for microbiological analyses.

of the hole, drilling fluid recirculation was stopped and the sampler drilled dry into the basal sediments. The corer was a single pipe 10 cm × 2.5 m that gave typical recoveries >1.5 m length. The sampler allowed recovery of large samples of considerable diameter so that the paring of two-thirds of the sample resulted in several kilogrammes of sediment acceptable for microbiological analyses. In latter experiments, a hydraulic paring device was constructed which used a plunger and hydraulic pressure so that in one operation the sediments were removed and forced through the mechanical paring device. The Phosphate barrel proved to be the most versatile of all samplers and was capable of recovering most sediment types except unconsolidated sands.

Field studies

Undisturbed sediment cores were removed from the sampler and the core liner was immediately carried into the Mobile Microbial Ecology Laboratory (MMEL). The facility is a 12-m self-contained mobile laboratory with full microbiological capabilities. Sediments were extracted from the core liners using an extruding device (Model P-107, Soiltest, Evanston, Illinois). Sediments exiting the core liners automatically entered a N_2-flushed glove bag (Coy, Ann Arbor, Michigan) slotted for two pairs of gloves, core extruder, and sample removal port. To minimize contamination during processing, the bag was disinfected prior to each use and ethylene-oxide-sterilized gloves were placed over the latex gloves of the glove bag. All paring and sample manipulations occurred in the glove bag. Using alcohol-flamed sterilized tools, approximately half of the outermost portions of the sediment core was pared away. After paring, the microbiological sample was placed into a flame-sterilized pan, quartered, mixed, and disbursed into sterile whirlpak bags. Bags were weighed, placed into quart canning jars, flushed with N_2, sealed, and removed from the glove bag through the airlock. The entire operation was completed within 30 min from the time of collection. Between each sample, the bag was cleaned, alcohol-wiped, and daily dismantled for cleaning and disinfection.

Subsamples were immediately available for pore-water chemistry measurements, initiation of onsite activity experiments, frozen for lipid analyses, prepared for shipment via overnight express to program participants, or archived. Cores exiting the ground by 14:00 were prepared for shipment by packing on ice, bagged, and boxed and were in transit by 16:00. In >90% of the instances, samples arrived in the appropriate laboratories across the USA by 10:00 the next morning.

Quality assurance

The onsite geologist, provided by PSI, maintained the lithological logs and compared logs from the microbiology hole with the observed lithologies and color photographs of cores collected from the preliminary hole. The geologist ensured that samples were collected at the correct depth, formation, and structure. In addition, the geologist monitored drilling procedures, drilling fluid viscosity, and depth of the hole. The choice of sampling tool was determined by the driller, geologist, and microbiologist.

Chemical tracers were used in the drilling muds as an indicator of drilling mud penetration into sediment samples. Potassium bromide was added at 900 $mg \cdot l^{-1}$ of drilling fluid as a conservative tracer and was assayed by ion chromatography with detection limits of 10 $\mu g \cdot l^{-1}$ pore water (Frederickson et al., manuscript submitted).

Potassium was present in some natural pore waters at concentrations of $0.3-5.0\ \mu g \cdot ml^{-1}$ limiting its use to a qualitative measure of drilling fluid contamination of recovered sediments. Bromide was below detectable limits in untreated pore waters and could be measured at 10^4-10^5 dilution of drilling fluids or as ppm contaminants when used as a conservative tracer for drilling fluid contamination in recovered sediments. Rhodamine, a fluorescent dye, was added at 20 $mg \cdot l^{-1}$ of drilling mud as an independent tracer and was assayed onsite to insure that highly contaminated materials would not be disbursed to participating labs. Rhodamine was assayed by mixing equal volumes of water with pared sediments and examining the supernatant by fluorometry using an excitation wavelength of 546 nm with fluorescence measured at 590 nm using a G. K. Turner fluorometer (Model 430, Palo Alto, California). Detection limits were $<1.0\ ng \cdot ml^{-1}$ supernatant.

Total bacterial plate counts were performed on each pared sample and drilling fluids using trypticase, yeast extract, and glucose pour plating media with 10 and 0.1 g of each C source $\cdot l^{-1}$ of medium to monitor the level of microbial colony-forming units (CFUs) in drilling fluids and in each sample. Additionally, total bacterial plate counts were determined independently from which colonies were picked and 22 biochemical tests were performed on each isolate to establish whether the microorganisms were similar to those of other formations or drilling fluids (Balkwill, manuscript submitted).

Results and Discussion

Obtaining aseptic, undisturbed, subsurface sediments from depths of 300 m proved to be a formidable task. Sample collection systems, which core sediments by hydraulic pounding or hollow stem auger, are difficult to use at depths >50 m partly because the drill rods absorb most of the shock, and such systems are poor at coring dense and compacted clays. Air drilling was eliminated due to likely contamination of the samples with O_2. Rotary drilling with bentonite viscosifier drilling fluids was the method of choice. Several sampling tools were required to retrieve the various types of subsurface materials examined in these studies. As shown in Fig. 2, four types of corers were used to obtain the subsurface sediments. Although the Dennison corer had a smaller diameter, it was best suited for unconsolidated sands. It was the only tool that used a retaining basket in order to keep cored materials from falling out of the core liner, and consequently was the only corer that could be used to retrieve sands. Limitations of the Dennison corer included small sample size and the clogging of the retaining basket which led to poor recoveries of clayey sediments. Quality assurance procedures often revealed >10 mg drilling fluid contamination $\cdot kg^{-1}$ sediments using the Dennison corer. The Dennison corer rarely collected >4 kg sediments of which 1–2 kg met the quality control specifications for microbiological studies.

The Shelby and Pitcher tube samplers were similar in that the core liners were exchangeable between the two tools. Both systems collected 3–10 kg silt or clay sediments with aseptic recoveries between 2–5 kg. The main difference between the samplers was in core liner protection. The Shelby system was well suited for less consolidated sediments since it used the tip of the core liner as the drilling bit. In compacted or hard materials, the Shelby system had a success rate of 33% for obtaining

satisfactory materials since the core liner became bent or torn (Table 1). The Pitcher barrel gave excellent recoveries of clays since the core liner was protected with a steel outer barrel. In less consolidated sediments, the drill bit likely led to more churning of the sediments, drilling fluid contamination, or loss of the sediment which resulted in a 64% recovery in silty sands.

The greatest percentage of successful recoveries and the largest quality assured sample size was obtained using the Phosphate barrel. Similar to the Pitcher and Shelby systems, sediments were required to have some silt or clay structure to be retained in the sampler because no core catcher was available. Advantages of the system were the 10 cm diameter of the core and the ability to use the sampling tool as the drilling bit. The major disadvantage was the lack of core retention and poor availability of replacement parts. Success of the tool required the mud flow valve to seal. The valve held the pressure of the drilling fluids contained within the string of drilling rods. Whenever sand disrupted the seals, the pressure from the drilling fluid within the 200 m of drilling rod pushed against the retained sediments and in the course of pulling the drill string to the surface the sample could dislodge from the Phosphate barrel.

Subsurface sediment recoveries for well P24 are shown in Table 2. The poor recovery of the Dennison sampler was demonstrated at 259 m depth where total recovery was 3.4 kg. Over one-half of the 3.4 kg was discarded during paring leaving 1.4 kg to be distributed among the investigators. Adequate recoveries were obtained from the Pitcher barrel samplers; total recovery averaging 6.7 kg and an average of 2.2 kg·sample^{-1} disbursed to the investigators. The Phosphate barrel provided excess materials and the investigators received nearly twice the requested amount.

Mechanical paring devices, as used in unconsolidated sands [12] to remove the outermost sediment layers, were not successful in these subsurface sediments. It proved impossible to force sediment materials from some core liners, and attempts at extrusion through the paring devices resulted in the destruction of core liners. Several samples required cutting the core liners into 0.3-m segments prior to extrusion. In the course

TABLE 1

UTILITY OF SAMPLING TOOLS IN VARIOUS SEDIMENT LITHOLOGIES

Sample tool	Number of attempts (% of attempts with acceptable samples)				
	Sand (>85% sand)	Silty sand (>10% sand)	Clayey (>10% clay)	Clay (>40% clay)	Total
Dennison	8(75)	6(50)[a]	3(33)	2(50)[b]	19(58)
Pitcher	9(33)	11(64)	9(56)	3(100)	32(60)
Shelby	ND	2(100)	ND	3(33)[c]	5(60)
Phosphate	ND	8(63)	6(83)	3(100)	17(76)[d]

ND, not determined.

[a] Clays often clogged cutting boot blocking sample recovery.

[b] Extremely difficult to extrude.

[c] Compacted clays bend or break the unprotected core liner.

[d] Does not include equipment failures due to unavailability of replacement parts.

TABLE 2

SUBSURFACE SEDIMENT RECOVERIES AT WELL P24

Depth (m)	Sample tool	Total recovery (kg)	Pared recovery (kg)	Percent of recovery disbursed
0	Shovel	10	6	100
34	Phosphate	>25	5	100
48	Phosphate	>25	10	100
61.3	Phosphate	>25	>7	100
96.5	Pitcher	10	4	75
125	Phosphate	>25	>7	100
147.5	Pitcher	10	2	50
154	Phosphate	>20	6	100
191	Phosphate	20	6	100
212	Pitcher	3	1.7	43
215.5	Pitcher	7	2.6	65
250.7	Phosphate	10	4	100
259	Dennison	3.4	1.4	35
269.3	Pitcher	3	2	50
277.4	Pitcher	7	3	75

of the three wells, the extruding device became worn requiring the development of a hydraulic extrusion system capable of extruding and paring compacted sediments.

It is desirable that sediment samples used for microbiological analyses in subsurface microbiology studies be free of drilling fluid contamination. No drilling operation to date allows for the sterile collection of deep terrestrial subsurface samples. Even aseptically collected sediments result in some level of contamination by the very nature of coring, recovering, extruding, and disbursing undisturbed subsurface sediment samples. A goal of the sampling program was to incorporate methodologies to determine the extent of contamination and to minimize the level of such contamination.

The data in Table 3 show the six quality assurance protocols used in this study. Upon

TABLE 3

QUALITY ASSURANCE OF MICROBIOLOGICAL SAMPLES FROM SUBSURFACE SEDIMENTS

Test	Performed by	Detection range	Protection factor
Lithology	Onsite geologist	NA	NA
Rhodamine	SPO[a]	$5\ \mu g \cdot l^{-1} - 50\ mg \cdot l^{-1}$	$10^4 - 10^5$
Total CFUs	Several microbiologists	$< 10^2 - 10^8$ CFUs $\cdot l^{-1}$	$10^1 - 10^6$
Ammonium	Independent analytical chemists	$10\ \mu g \cdot l^{-1} - 100\ mg \cdot l^{-1}$	$10^2 - 10^4$
Bromide	Independent analytical chemists	$10\ \mu g \cdot l^{-1} - 1000\ mg \cdot l^{-1}$	$10^2 - 10^5$
Potassium	Independent analytical chemists	$10\ \mu g \cdot l^{-1} - 1000\ mg \cdot l^{-1}$	$10^2 - 10^5$

NA, not applicable.

[a] Sample procurement officer, onsite microbiologist.

[b] Onsite microbiologist and Balkwill (manuscript submitted).

[c] Fredrickson et al. (manuscript submitted).

extrusion of the sediment material from the core liners, an onsite geologist verified that the retained materials were from the selected location. This was accomplished by comparing sediments with known properties of the formation determined the week before in the preliminary hole. The preliminary boring was cored continuously in a nonaseptic manner suitable for geologic studies and logged geologically for both lithology and stratigraphy. In addition to written logs, descriptive color photographs were taken of each formation to be sampled. By comparing retrieved sediments with the descriptive logs and photographs, the sediments were collected from the prescribed locations.

A second field protocol was the examination of each of the sampled cores for the presence of rhodamine dye. If no dye was visually observed, scrapings of the aseptic material were diluted with equal volumes of water and the supernatant assayed for the presence of the dye by fluorometry. Rhodamine dye analyses were performed in the field and provided a quality assurance protection factor of $10^4 - 10^5$ or $\approx 10 - 100$ mg drilling fluids $\cdot$ kg^{-1} sample. If the sample was from the correct formation, and free of rhodamine dye – both visually and fluorometrically – then the materials were distributed for analyses.

All other quality assurance protocols were performed offsite. Total colony-forming units (CFUs) on bacterial plate count media were performed on each sample and on the drilling fluids. Freshly prepared drilling fluids contained <100 microorganisms $\cdot$ ml^{-1} whereas drilling fluid which had been recirculated through several aquifers, contained CFUs approaching $10^6 \cdot$ ml^{-1}. Isolates from each sample location were screened for 22 biochemical and physiological tests. As a general rule, an isolate from one location was rarely seen at any other location in that core hole or in another boring (Balkwill, manuscript submitted; Fliermans et al., manuscript submitted). In addition, the sediments exhibited CFU enumerations of $<10^2 - 10^8 \cdot$ g^{-1} and often were 10^6 greater than CFUs of drilling fluids. Ghiorse (pers. comm.) observed a small flagellated protozoan (2.5×8 μm), *Bodo* spp., present in the drilling muds at $50 - 500$ times more abundant than any other protozoan, but it was only observed in the surface samples and never in the samples collected from the deep subsurface sediments.

The remaining quality assurance protocols were determined through pore water chemistry analyses. Ammonium was present in the bentonite drilling fluids, while potassium bromide was added as a conservative ionic tracer. All three species were determined to be near-background levels in the subsurface sediments (Frederickson et al., manuscript submitted). The ionic tracers provided protection factors against drilling fluid contamination of $10^4 - 10^5$ (Table 3). The combination of quality assurance protocols in the field along with independent laboratory verification resulted in a comprehensive program to quantify and limit contamination from drilling operations. The importance of the quality assurance program was highlighted in clay sediments which often contained $<10^2$ CFUs $\cdot$ g^{-1} although drilling fluids contained $10^2 - 10^5 \cdot$ g^{-1}.

Three major sources of possible contamination were drilling hardware, drilling fluids, and sample processing. Processing of samples in the disinfected and N_2-flushed glove bag using ethylene-oxide-sterilized gloves and flame-sterilized utensils adequately reduced contamination during paring and disbursement of samples

into sterile bags. Contamination from drilling hardware was best reduced by steam-cleaning and use of samplers of large diameter and longer length. Reducing contamination from drilling fluids was a formidable task. The goal was to obtain a large sample in front of the drilling-mud sediment interface. The Phosphate barrel was the best sampler in sediments containing clays or silts with a stiff sediment structure. The Dennison sampler was the least effective but allowed collection of loose sands. Combination of rhodamine as an onsite quality control parameter with independent measures of ionic tracers proved satisfactory at obtaining sediments which were minimally contaminated by drilling fluids.

The protocols listed here are capable of providing multiple measures of 10 $mg \cdot kg^{-1}$ contamination but lack the sensitivity to ascertain $pg \cdot kg^{-1}$ levels of contaminants. The combination of these protocols with the finding that microorganisms did not appear in multiple geological formations, strengthens the assertion that subsurface sediments were collected with minimal contamination and that the observed microorganisms were in fact from subsurface habitats rather than from drilling contamination.

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