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Effects of Impact Cratering on the Microbial Biosphere of the Deep Terrestrial Subsurface

Thesis

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Effects of Impact Cratering on the Microbial Biosphere of the Deep Terrestrial Subsurface

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Abstract

The 2005 ICDP-USGS deep drilling of the Chesapeake Bay Impact Structure (CBIS) returned the first complete core through an impact structure. A strict set of contamination assessment measures were implemented during sample collection to ensure that materials could be confidently used in geobiology, molecular biology and microbiology studies. Through direct cell counting, culturing and molecular analysis, samples offered a unique opportunity to characterize the subsurface microbial community present at depth in an impact structure. This work outlines how subsurface habitats can recover after impacts, and how impacts act to generate new microenvironments where microorganisms can colonize.

Geobiology studies revealed a pattern of microbial abundance that corresponds to lithological transitions within the crater structure. Three 'zones' of abundance were defined, with the first showing a steeper logarithmic decline in cell numbers than seen in other deep subsurface environments. This is followed by a zone of cell numbers below the detection limit of the methods used. Finally, the deepest section of the core shows an increase in cell numbers, indicating that recolonisation has occurred following the impact event.

Culturing studies were consistent with the results of enumerations, with successful cultures retrieved from microbiological zones 1 and 3. The majority of cultures were acquired using heterotrophic media, although cultures were also returned with media for iron reducers, iron oxidizers, sulfate reducers and humic acid utilisers. Culturing studies and molecular studies showed that a diverse consortium of microorganisms is present in the deep subsurface of the CBIS. Finally, the ability of microorganisms to access nutrients and minerals from meteoritic material was analyzed. The results of these studies add to our knowledge of how impacts events can affect subsurface microbial habitats; both directly by kinetic disruption of the environment, and through the delivery of exogenous materials.

EFFECTS OF IMPACT CRATERING ON THE MICROBIAL BIOSPHERE OF THE DEEP
TERRESTRIAL SUBSURFACE

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Figures and Tables

Chapter 1: Introduction to Geomicrobiology and the Chesapeake Bay Impact Structure

1.1 ABSTRACT

In September of 2005, a large-scale research project supported by the Intercontinental

Drilling Program (ICDP) and the United States Geological Survey (USGS) was begun to

collect a continuous drill core from the surface to the crystalline basement underlying the

Chesapeake Bay Impact Structure (CBIS). This crater is an 85 km- diameter structure

formed by an impactor that struck the Earth roughly 35 million years ago. The CBIS is

centred near the mouth of its namesake, the Chesapeake Bay, on the eastern shore of the

United States of America. The nature of the impactor that formed the CBIS is unknown,

but the resulting crater is now one of the best-preserved crater structures known on Earth

today. The structure itself is completely sub-terrestrial, with no features discernible at the

Earth's surface.

Deep drilling of the CBIS was performed in three coring efforts, together spanning

from the present-day Earth surface down to near the base of the structure, and totalling

1766 m in length. Drilling began in September of 2005 and the primary drilling effort was

completed in December of the same year. The project returned one of the most complete

cores ever retrieved from an impact structure, and was the first such core where

assessments of biological contamination were performed in order to allow for

microbiology and molecular biology analysis of core materials, described in this thesis.

1.2 Introduction

In September of 2005, an ambitious project to retrieve a contiguous core from the

terrestrial surface to the basement rock underlying one of the largest impact structures on

Earth was begun. The 2005 Intercontinental Drilling Program (ICDP)-United States

Geological Survey (USGS) deep drilling of the Chesapeake Bay Impact Structure provided

the most complete section through an impact structure to date, with three coreholes

retrieving a continuous core of ~1766 m (Gohn *et al.* 2006). The project brought together

researchers across a range of disciplines and representing eight countries.

The Chesapeake Bay Impact Structure (CBIS) is a large impact structure estimated

to be 35.5 million years old and is located on the eastern shore of the Commonwealth of

Virginia in the United States of America. Today, the structure is entirely subterranean and

is positioned beneath the mouth of the Chesapeake Bay. The CBIS has an average diameter

of 85 km (Horton *et al.* 2005b, Horton *et al.* 2006; Horton and Izett 2005), and the outer

rim extends from inland Virginia out into a small part of the western Atlantic Ocean

(**Figure 1**). It is the largest known impact structure in the United States (Horton *et al.*

2005b). The centre of the structure is located near the town of Cape Charles, VA, some

320 kilometres south of Washington D.C. and 65 kilometres north of Newport News, VA

(**Figure 1**).

The CBIS is a unique site for study because it is a rare and extremely well

preserved shallow-marine impact site. Although the impact event occurred roughly 35.5

million years ago in the late Eocene, the effects of the impact on the subsurface geology

and hydrology of the region have been long-lasting. Changes in the flow of groundwater

have altered sources and quality of water for the roughly 2 million people who now live

within the region. The impact that formed the CBIS may actually be responsible for much

of the geological formation of the modern Virginia seaboard (Poag 1995).
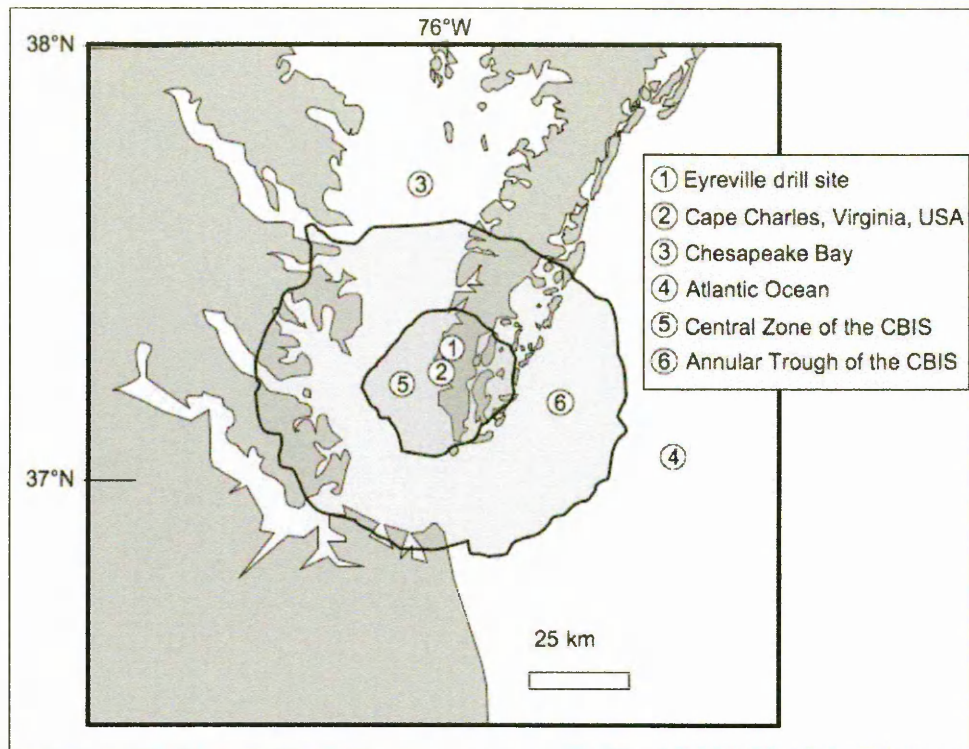
Figure 1 - Location of the Chesapeake Bay Impact Crater. The outer rim and central crater are outlined to highlight the central zone (5) and annular trough (6) of the crater structure. This figure is based on data from Horton *et al.* (2005b).

1.2.1 History and Formation of the CBIS

The CBIS was formed by a massive impact that occurred some 35.5 million years ago in a period of Earth's history known as the late Eocene epoch (Gohn *et al.* 2008; Larsen and Crossey 2004). The late Eocene was a period characterized by warm global temperatures and high sea levels. Life on Earth was complex, with mammals roaming under the cover of dense tropical rainforests that lined the eastern coast of what is now the USA. Most of the area we think of as coastal Virginia today was actually underwater in the late Eocene, with the Atlantic shoreline resting more than 60 km inland from its present-day position.

The impact that formed the CBIS caused a massive disruption in the geological formations of the Atlantic coastal plain. Generally, the Atlantic coast of the present-day United States was formed through a continual process of marine deposition and erosion (Poag 1997). Subsiding sea levels over a roughly 200 million-year period left regular deposits of layered marine sediments (i.e. Owens and Gohn 1985; Ward and Strickland 1987). The amount of sediment that was deposited varied along with paleoclimate

conditions and pulses of Appalachian[1] tectonic activity (Poag 1997). In the region of the CBIS, this regular layering of sediments lies over a gently sloping basement of crystalline rock known as the 'Salisbury embayment', which sits on the western edge of the Baltimore Canyon Trough (**Figure 2** and **Figure 3**). These regularly layered sediments, which were deposited over hundreds of millions of years, were then disrupted and re-arranged in the region specific to the CBIS by the crater-forming impact. The target material (i.e. the rocks that were struck by the impactor to form the CBIS) had been previously characterized by seismic, gravity and magnetic surveys prior to 2005 ICDP-USGS drilling program (Poag 1997).
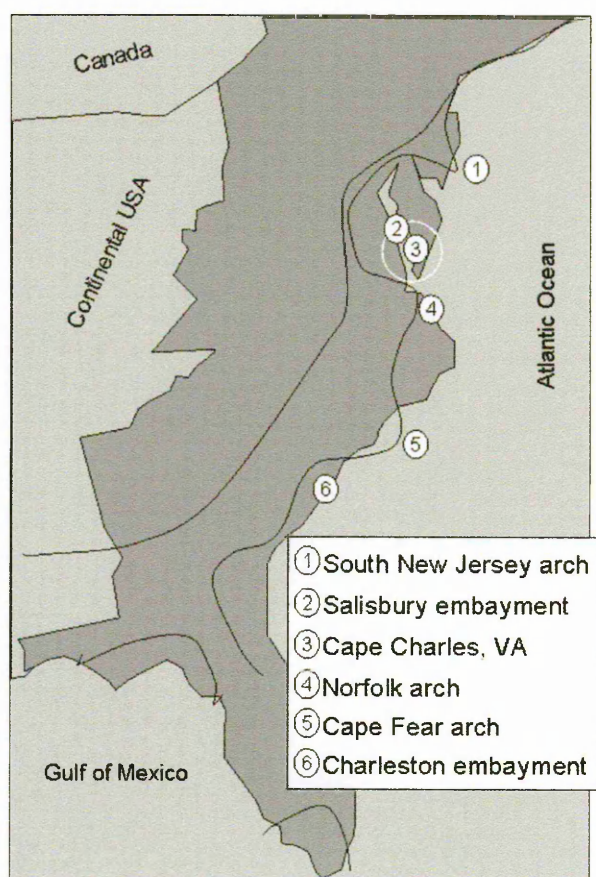
Figure 2 - Select geological features of the Atlantic Coastal Plain, including those of the Virgnia Coastal Plain near the Chesapeake Bay Impact Structure. The general location of the crater structure is outlined in white. Geological information is adapted from Ward and Powars (2004).

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[1] The term 'Appalachian' refers to the region encompassing the Appalachian Mountains in the eastern United States. The mountain range lies toward the western regions of the state of Virginia, inland from the Virginia coastal plain.
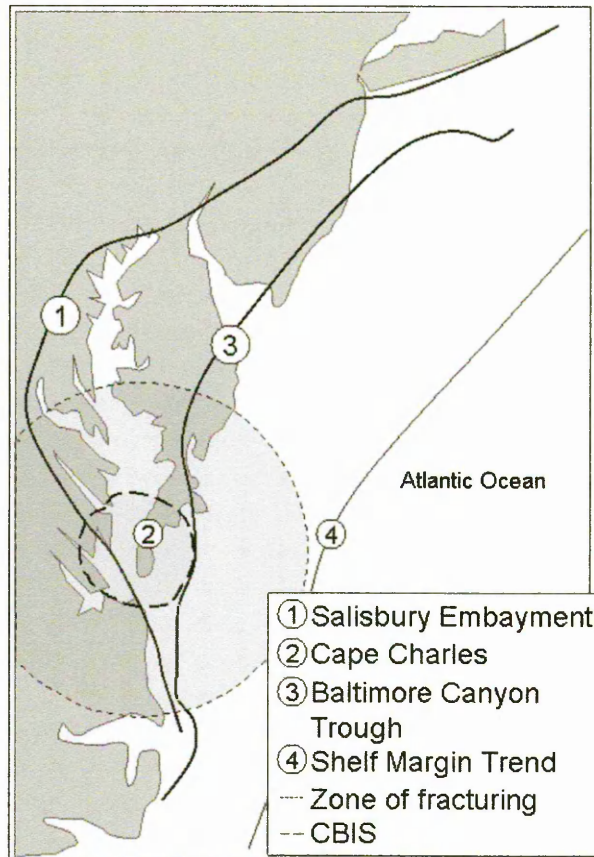
Figure 3 - Closer view of geological features in the region of the CBIS, including the location of the Baltimore Canyon Trough. Derived from Prather (1991) and Poag (1997). This figure shows geological features of the Atlantic Coastal Plain in relation to the location of the CBIS. Additionally, a zone of fracturing near the surface of the Earth occurred, and is outlined. This fracturing is thought to be related to the impact forces that formed the CBIS (Poag 1997).

The impact was a first order *convulsion* event, which is defined as an extraordinarily energetic geologic event with regional influence (Clifton 1988). The Chesapeake impact is thought to have occurred near the same time as the similarly-sized Popigai impact in Siberia. A regional tektite strewn field is believed to be associated with the CBIS (Koeberl *et al.* 1996; Poag *et al.* 1994), commonly referred to as the North American tektites (Koeberl *et al.* 1996). Tektites are, in short, small fragments of round or irregularly shaped natural glass thought to form when melted materials are ejected by an impact and subsequently re-solidify in the atmosphere as they fall back down to the surface of the planet. Their unique shapes make them easy to identify, and the amount and distribution of tektites over the surface can be used to trace them back to their point of origin and ultimately identify the original impact site (i.e. Koeberl *et al.* 1996).

It is not known what class of object formed the CBIS, and because of this, the impactor itself is often referred to as a *bolide*. This is a generic term used to represent all crater-forming objects, such as asteroids, comets and meteorites (Greeley 1994). The global impact of the Chesapeake Impact event is little-known. There is some debate concerning whether or not the event could have contributed to global cooling at this period in Earth's history (Larsen and Crossey 2004); and a dispute in the literature over whether or not the CBIS-forming impact could have been linked to any local extinction or mass extinction events at the time (i.e. Poag 1995, 2002; Walkden and Parker 2008).

The CBIS is considered a *complex crater*, meaning that its structure is more complex than a simple bowl-shaped indentation. Today, the entire crater structure is buried some 300-500 m below the Chesapeake Bay and its surrounding peninsulas (Poag 1997). On average, the entire structure is roughly 85 km wide (Collins and Wunnemann 2005; Gohn *et al.* 2006). It is somewhat difficult to determine the exact width of the crater due to the fact that it was formed in shallow marine conditions. In aqueous conditions, and with the movement of ocean waters, a large amount of 'slumping' at the crater edges could have occurred. This means that materials from the crater walls would have caved into the crater structure itself, likely extending the size of the crater's outer rim. The CBIS also contains a deeper, central crater (referred to as the 'inner basin') that is estimated to be either 30-38 km wide and subquadrate in shape (Powars and Bruce 1999; Powars *et al.* 2005), or alternatively 35-40 km wide and 'irregular' in shape (Poag *et al.* 1999). Seismic data suggests that this inner basin of the CBIS penetrates into crystalline basement rock that sits below the marine sediments of the late Eocene seafloor into which the impact occurred. The marine sediments persist to a depth of roughly 1.6 km below the present-day sea level (i.e., 1.3 km below the outer rim of the crater) (Horton *et al.* 2005a,b). At the very centre of the crater is a structure known as the 'central uplift'. This is a mass of crystalline rock with a diameter of about 15-20 km that was forced upward, or 'bounced back', after the impact and rises out of the crater floor (Horton *et al.* 2005a). Surrounding the inner basin is a flat-

floored region of about 24 km wide named the 'annular trough' (Poag *et al.* 1994). Outside of the CBIS is a fracture zone of about 35 km in width, which contains various faults and fractures created by the force of the impact (Horton *et al.* 2005a, b).

The features of the CBIS discussed were formed in a brief and powerful event that disrupted a geologic profile established over hundreds of millions of years of natural environmental processes. Schematics of what the Atlantic Coastal Plain in the region of the CBIS looked like before the impact and how they appear now are shown in **Figures 2** and **3**. A generalized stratigraphic chart of the region can be seen in **Figure 4**.

The entire structure of the CBIS was covered and subsequently preserved beneath a 150-400 m thick blanket of post impact sediments (Poag *et al.* 1994; Powars and Bruce 1999; Horton *et al.* 2005a, b). In the period of time that followed, the crater continued to be covered with sediment deposits and earth as sea levels subsided. Today, no major identifying outcrops are visible at the planet's surface.

As early as the 1900s, researchers began noticing that there was something unique about the region of the Chesapeake Bay, and specifically the area now known to contain the CBIS (Sanford 1913). However, the crater itself was not discovered until the end of the 20th century, when various lines of evidence were combined and new methods were used to determine its existence and specific location. In 2000, the Chesapeake Bay Impact Crater Project, a dedicated multidisciplinary effort, was initiated to define and study the unique site.
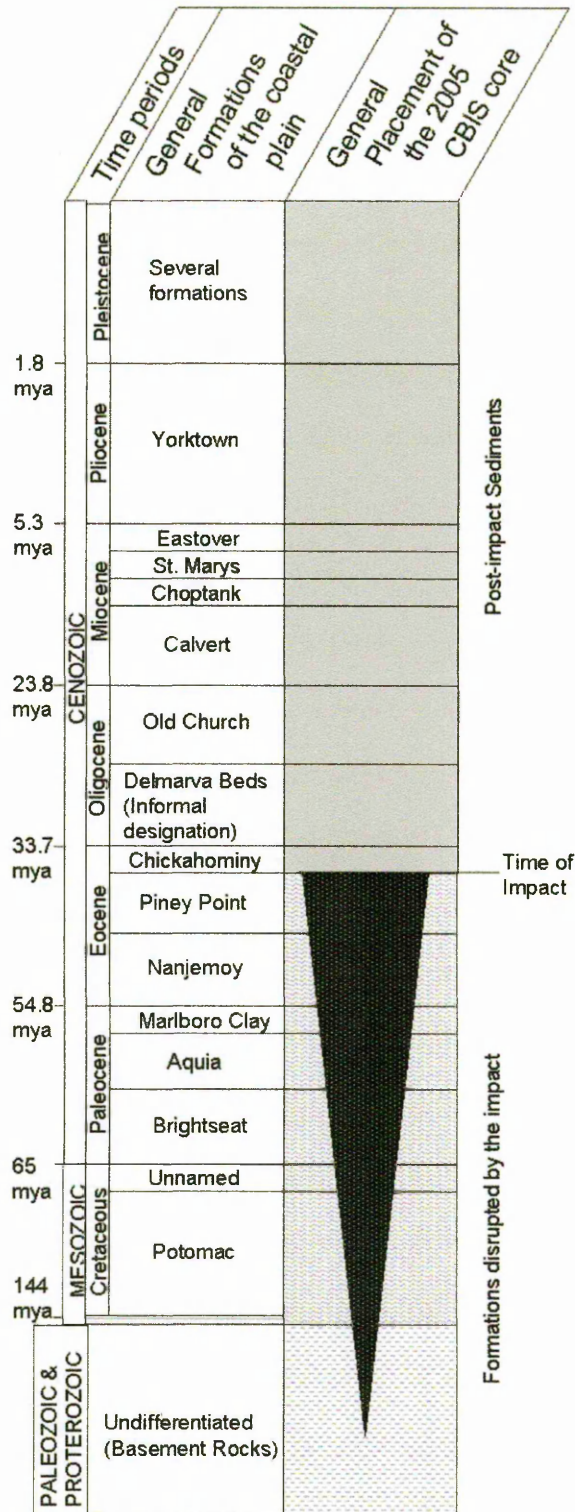
Figure 4 - This image shows the placement of the CBIS in relation to the general stratigraphy of the Virginia Coastal Plain. Post-impact sediments have built up since the impact ~35 million years ago (mya). The impact is thought to have affected materials down the basement rocks, and older than the Cretaceous period (>144 mya). Data derived from Gohn *et al.* (2006), Poag (1997), Thomas *et al.* (1989) and Ward and Strickland (1985).

To this day, the exact process behind the formation of the CBIS is still not

understood, and the story will likely change or adapt as research efforts continue. Studies

over the past decade have provided enough information to construct a general framework for the event. The CBIS is sometimes referred to as an 'inverted sombrero' because of its shape, which likely resulted from the contrast between the hard crystalline basement rock and the overlying, water-saturated marine sediments into which the bolide impacted (Kenkmann 2004).

The bolide impactor plunged to Earth and collided with the shallow Atlantic Ocean of the late Eocene. The target material can be divided into three general layers: seawater ranging from 0-310 m deep, clastic sediments or unconsolidated shallow marine sediments, and the underlying crystalline metamorphic and igneous basement rock (Horton *et al.* 2005a) (**Figure 5**). The bolide is estimated to have been roughly 3 km in diameter and struck its target with a velocity of around 20 km per second (Horton *et al.* 2005a; Powars *et al.* 2008). Of course, such estimates can vary depending upon on the assumed composition of the impactor (asteroid, comet, etc.). The bolide was vaporized completely along with water and material in the shallow ocean, marine sediments and crystalline basement rock. Initially, a crater of roughly 35 km wide was formed. The energy of the impact generated massive tsunami waves that pushed outward from the point of impact (Poag 2004b), and which left a cavity in the ocean water as well as the underlying sediment and rock (**Figure 5**). Melted material was ejected from the crater and solidified in the atmosphere to form raindrop shaped droplets known as tektites (**Figure 5**). These solid droplets then rained down to the Earth to form the tektite fields, which are strewn as far away as Texas and provided early clues to the crater's existence (Horton and Izett 2005; Koeberl *et al.* 1996).

The crater floor then rebounded, causing slumping and collapse of the crater rim. This formed the annular trough (**Figure 5**) and extended the crater's diameter to the roughly 85 km we see today. Today, the inner basin remains 500-1000 m deeper than the annular trough formed by the collapse of the crater rim (Kenkmann 2004). More of the ejected material then fell back into the crater along with ocean water that rushed back to

fill the cavity. This deposited an assortment of debris into the crater structure, ranging from microscopic particles to house-sized blocks of granite (Edwards and Powars 2003). The material deposited in this step of the CBIS formation is what we now call the 'resurge breccias' (Larsen and Crossey 2004). Some studies have indicated that this resurge may have been more debris-loaded than previously studied marine craters on earth (Ormo and Lindstrom 2000; Ormo *et al.* 2004). The incoming material that poured into the crater would have met and reacted with thermally heated brine at the basement of the crater. Temperatures in some sections of the core are likely to have reached levels sufficiently high to kill any organisms known today, and would have sterilized materials in these regions of the CBIS (Sanford 2003). Elevated temperatures as a result of the impact could have persisted for as long as 1 million years (Sanford 2003). A thick sedimentary layer then settled out of the turbulent ocean waters and accumulated on top of the crater structure after the fallout materials settled. This sediment layer preserved the crater at the mouth of the Chesapeake Bay until modern times. This accumulation of sediment on top of the CBIS is also one reason that no major outcrops from the crater can be identified at the surface today. Over the past ~35 million years following the impact event, land mass continued to accumulate with the ebb and flow of ocean levels. Today, the CBIS is completely buried beneath the modern land surface present in eastern Virginia (**Figure 5**).

One of the most important features that helped identify the CBIS in modern times was a unique disruption of sediments that comprise an extensive system of underground aquifers and confining units for groundwater across the coastal plain (McFarland and Bruce 2004; 2006). The disruption of coastal plain groundwater reserves is confined to the Coastal Plain of Virginia. As humans began to permanently settle the region, wells were dug for drinking water. As early as the 1800's, residents discovered that a region of salt water was present in groundwater reserves, and this region extended as much as 50 km inland from where it would have normally been expected based on the proximity of the ocean (McFarland and Bruce 2004, 2005). This disruption in the pattern of groundwater in

the Coastal Plain of Virginia was further identified through sediment-cores and well sites and dubbed the 'inland saltwater wedge' (Sanford 1913). Today, the inland saltwater wedge can now be traced to correspond along the margin of the CBIS.
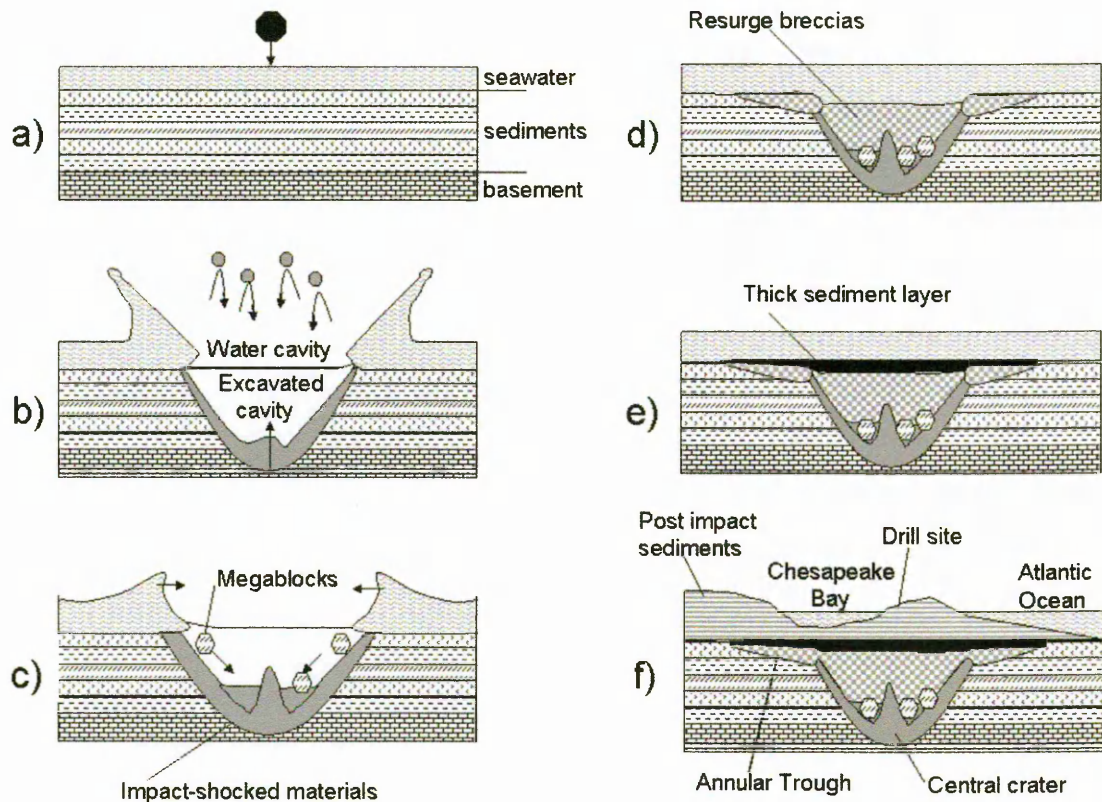
Figure 5 - General representation of the formation process of the CBIS (diagram not to scale). a) The bolide impactor struck shallow seawater off the east coast of North America. b) The force of impact generated a crater in the seawater and underlying marine sediments. Materials were ejected from the crater, some of which fell back in to partially fill the base. At the centre of the crater, materials rebounded to form a central uplift feature. c) Materials including giant megablocks of granite caved in from the crater walls, extending the size of the structure. Tsunami waves rushed back to fill the crater. d) Tsunamis deposited materials into the crater, filling the structure with resurge deposits. e) A thick layer of sediment settled on top of the structure. f) The CBIS today. Over ~35 million years following impact, post impact sediments collected to cover the crater and form the land we see today.

The saltwater wedge phenomenon is likely the results of the massive amount of seawater deposited into the crater structure shortly after impact as opposed to the evaporation of water caused by thermal heating (McFarland and Bruce 2004). This is evidenced by the bromide-to-chloride ground-water concentration ratio of water in the saltwater wedge, which corresponds to the ratio characteristic of seawater (McFarland and Bruce 2004). Enrichment of bromide in the saltwater wedge has been attributed to the decay of organic materials washed into the crater along with the seawater and sediment,

and possibly, in a small part, to evaporation caused by thermal activity of the brines initially present at the bottom of the crater that were subsequently re-diluted into the seawater that surged to fill the crater shortly after formation (McFarland and Bruce 2004). It should be noted that this is a theory for enrichment of bromide that has not been definitively proven. Additionally, ratios of chlorine 36 to total chloride in ground water retrieved from the USGS-NASA Langley core (Horton *et al.* 2005b) support the idea that the CBIS contains seawater. The seawater trapped within the CBIS has experienced limited mixing with neighbouring groundwater reserves, as evidence by monitoring the mixing of the two water types along the edge of the crater (Horton *et al.* 2005b). This is a further indication that the salinity in groundwater found in the saltwater wedge is directly associated with the CBIS. Today, the western rim of the crater structure can be used to demarcate a transition zone margin, where fresh groundwater sits to the west of the transition zone outside of the structure and saltwater rests to the east inside the CBIS.

The isotopic composition of water retrieved from the CBIS is also lighter than modern day seawater, a fact that has been used to indicate that the seawater within the CBIS predates the Pleistocene epoch of 2 million years ago (McFarland and Bruce 2004). The age of the groundwater close to the centre of the CBIS is several million years or more older than this based on studies of the USGS-NASA Langley core (McFarland and Bruce 2004). In contrast to the age of groundwater in the CBIS, fresh groundwater from outside of the crater has been dated to only 40,000 years using carbon 14 concentrations (McFarland and Bruce 2004). This is further indication that mixing between fresh groundwater outside of the crater and seawater within the crater at similar depths has been limited. Because of this, the CBIS could represent a subsurface environment that has been isolated from surrounding environments for a significant period of time. Replacement of seawater in the CBIS by flushing of fresh groundwater in the region over the past 2 million years is unlikely due to the low-permeability of crater-fill sediments as well as the clay-like material that capped the crater following resurge filling (McFarland and Bruce 2004).

This means that seawater remained trapped within the CBIS, while surrounding groundwater outside of the crater experienced flushing that extended in places "nearly to the edge of the continental shelf during the Pleistocene glacial maximum of 18,000 yeas ago" (McFarland and Bruce 2004). The general trend of water movement in aquifers on the Atlantic Coastal Plain is down-slope toward the sea, meaning that without the CBIS present, a large cache of saltwater would not remain in the subsurface for extensive periods of time.

The Chesapeake Bay Impact Structure is unique on Earth because of its size, location, the conditions of its formation and the lasting effect it has had on its surrounding environment. The structure was formed in shallow marine water, and due to the ways in which it has been preserved in the subsurface, it may represent the best preserved impact crater located on a predominantly siliciclastical[2] continental shelf known today (Horton et al. 2005b). The formation of the CBIS resulted in the creation of a unique, highly saline environment trapped within the crater structure itself due to non-porous fill material that disrupted the normal patterns of groundwater movement on the Coastal Plain of Virginia. The disruption effect of the CBIS on groundwater movement continues to this day.

The CBIS is located in an area experiencing high population growth, and its influence on groundwater supplies is of great importance in the future development of the region as urbanization continues. Industrial pumping centres are currently present across the Coastal Plain of Virginia; and there are now proposals for major increases in pumping and desalinization of brackish groundwater from within the impact structure. This is to meet the growing demand imposed by the rapidly increasing population and high levels of urbanization in the region. Understandably, the Commonwealth of Virginia is interesting in collecting data concerning the CBIS and its associated water resources in order to determine the best way forward for pumping activities, and how such activities will affect

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[2] i.e. non-carbonate sedimentary rocks such as sandstone that contain silica-bearing minerals like quartz

the movement of brackish groundwater out of and within the CBIS. Groundwater resources are of obvious economic importance to the Coastal Plain of Virginia, considering that they are the primary source of useable water, particularly in rural areas. In the year 2000, aquifers in the region supplied more than 100 million gallons of water per day to citizens, business and industries (Emry and Miller 2004). As the urban population continues to grow, groundwater will only become a more valuable resource and aquifers are already beginning to decline.

The isolated environment contained within the CBIS may provide a habitat for unique forms of subsurface organisms. These communities of microorganisms may have been isolated from the surrounding subsurface communities present outside of the CBIS for geological time periods, and could contain organisms that are distinct to the environment of the CBIS.

1.2.2 Studies Thus Far

Even though features of the CBIS, such as the inland saltwater wedge, had been identified early in the 1900s, it was not until 1996 that geologists from the USGS and the Virginia Department of Environmental Quality (DEQ) showed direct evidence for the existence of the CBIS (Emry 1999; Emry and Miller 2004). Seismic data that revealed the crater's rim was shown to match the contours of the inland saltwater wedge, providing the first indication that brackish groundwater could be attributed to an impact structure underlying the entire region. Local authorities found this information to be of great interest, because in Hampton Roads alone, a region of the Eastern Virginia Ground Water Management Area, thirteen public water utilities serviced a total of 1.6 million people. Five of these utilities withdraw and treat brackish groundwater and more such facilities are planned to cope with increasing populations in the area (Emry and Miller 2004).

After the local government was made aware of the CBIS in 1996, scientists developed defined phases of study that included drilling bore holes around the crater's edge (Emry and Miller 2004). In 2000, a full-scale research program specific to the CBIS began

with the multidisciplinary Chesapeake Bay Impact Crater Project. Currently, five holes have been drilled as part of this project, with the first being the 2000 USGS-NASA Langley corehole in the outer crater. The most recent corehole and subject of this study was the 2005 ICDP-USGS deep drilling project, completed in December of 2005 just north of Cape Charles, Virginia, near the crater's centre (**Figure 6**).
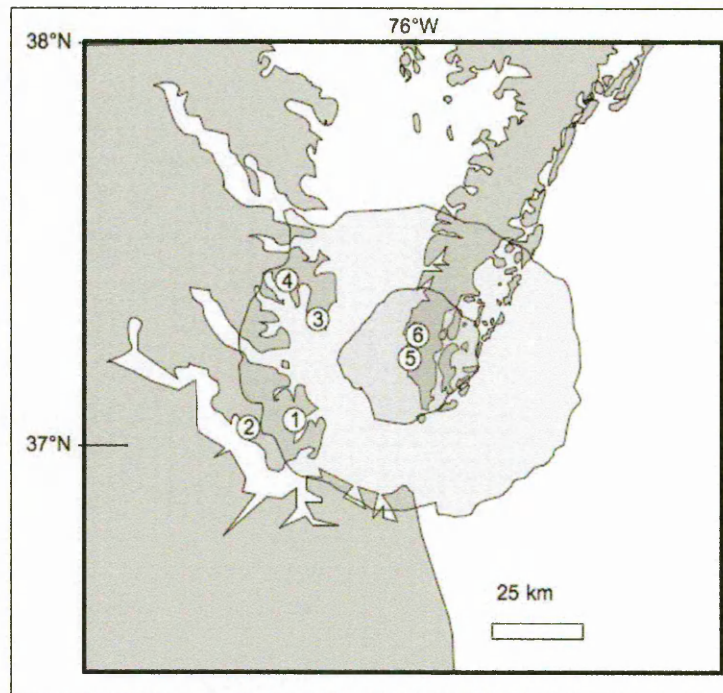
Figure 6 - Location of coreholes as part of the Chesapeake Bay Impact Crater Project. (1) USGS-NASA Langley corehole, in the western annular trough in Hampton, Va. 635.1 m total depth. Drilled in 2000. (2) USGS Dorothy R. Watkins Elementary School corehole, just outside the outer margin in Newport News, Va. 300.3 m total depth. Drilled in 2002. (3) USGS Bayside corehole, in the western annular trough on the Middle Peninsula at Bayside, Va. 728.5 m total depth. Drilled in 2001. Located 8 miles outside of central crater. (4) USGS North corehole, in the western annular trough on the Middle Peninsula, Va. 435.1 m total depth. Drilled in 2001 (5) Cape Charles test hole. 823 m. Drilled in 2005 (6) ICDP-USGS corehole, Eyreville Farm, VA. 1.76 km. Drilled in 2005. Located in the central uplift of crater.

The USGS-NASA Langley corehole was the first to penetrate the entire crater-fill section and uppermost crystalline basement rock of the CBIS, and provided a core section of the crater's annular trough (Horton *et al.* 2005b). This core provided the first comprehensive look at crater materials from the surface of the planet to the basement of the crater in a single location. The USGS-NASA Langley corehole was also accompanied by high-resolution seismic-reflection and seismic-refraction surveys, audio-

magnetotelluric[3] (AMT) surveys and research that was performed in a range of scientific disciplines (Horton *et al.* 2005a, Poag 2005; Catchings *et al.* 2005; Pierce 2005). The corehole reached a final depth of 635.1 m (Gohn *et al.* 2005) and proved that crater-fill materials were indeed preserved beneath the 235.6 meter-thick blanket of post-impact sediments covering the structure (Horton *et al.* 2005b). Within the core sections, biological remains were found including spores and pollen, dinoflagellate cysts, calcareous nannofossils, marine ostracodes and vertebrate remains (Frederiksen *et al.* 2005, Edwards *et al.* 2005). The next three coreholes of CBIS sediments were also located outside of the central crater and provided increased knowledge about the crater's three-dimensional structure and the interaction of fresh and salty groundwater near the crater's edge (Horton and Izett 2005). The collective data from these first four coreholes were used to define the location of the crater's buried outer margin (Powars *et al.* 2005).

The first set of drilling efforts provided scientists with an important look into the outer regions of the CBIS and returned invaluable information about its history and formation that prepared research for the next and current phase of the project. The 2005 ICDP-USGS deep drilling of the CBIS provided the first data from the deep trough surrounding the central uplift of the crater. New information concerning the size and characteristics of the inner crater, melt-rock formation and post-impact modification of materials was collected. Studying the inner crater and areas of the central uplift is important because the most complete sequence of impact-generated and post-impact deposits in craters has previously been shown to reside near the centre of such structures (Ormo *et al.* 2004).

Additionally, the ICDP-USGS corehole was the first drilling effort of an impact structure that implemented proper contamination assessment, providing for a complete study of the deep biosphere of the CBIS using modern microbiology and molecular

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[3] This is a method of imaging structures below the surface based on measuring natural variations in the Earth's electromagnetic field.

biology techniques (Gohn *et al.* 2006; Gronstal *et al.* *In Press* (a); see also **Chapter 2**). Impact-generated hydrothermal activity is also most easily studied in the central region of impact craters (Ormo *et al.* 2004), meaning that the associated biological studies in the unique environment are most useful near the crater's centre where hydrothermal energy could have produce a sterilizing pulse and evolutionary stresses for communities.

1.2.3 Subsurface Microbes

The relatively new science of geobiology has shown us the important interactions that exist between microbes and minerals on our planet. Microbes and minerals in the subsurface of the Earth react in interdependent ways, whereby microbes use various mineral types and sources like petroleum to derive energy and carry out metabolic processes (e.g. Johnson and Hill 2003; Liu *et al.* 1997; Wirtz 2006; Zhang *et al.* 2005). Studying microorganisms in the deep subsurface of our planet can yield valuable insights into how such communities relate to geology and mineral cycling inside the Earth. Mineral and petroleum reserves on our planet are valuable and limited resources, and understanding the role of microorganisms in degrading, maintaining and cycling these reserves is immensely important.

Subsurface microorganisms have evolved to make use of unique energy inputs for metabolism and to survive in distinct environmental niches. These microorganisms include species that can derive energy from minerals such as iron and sulphate, and others that can survive at extremely high temperatures (e.g. Liu *et al.* 1997; Zhang *et al.* 2005). Subsurface environments can vary greatly in the temperature, availability of water, pH and other conditions. Organisms inhabiting these environments are sometimes forced to survive under conditions of extremely low influx of nutrients and essential elements for growth.

Many subsurface organisms can, in theory, survive in the deep subsurface completely independent of the surface biosphere (i.e. Stevens and McKinley 1995; White *et al.* 1998). This has far reaching implications for the origin of life on our planet and the

potential for life on distant worlds. It has been suggested that subsurface environments could have been a crucible for life on early Earth (Farmer 2000; Kring 2000). These environments, while distanced from the energy of the sun, can provide protection from harmful conditions at the surface of celestial bodies. Subsurface environments also represent some of the most unique and relatively extreme environments on Earth. It has also been suggested that the common ancestor of life on Earth was an organism capable of surviving in extreme environments and was likely thermophilic (Woese 1987; Pace 1991). Additionally, processes common in the subsurface, like iron reduction, could have existed on our planet long before aerobic respiration (Liu *et al.* 1997; Nealson and Saffarini 1994).

Studying the interaction of microbes and minerals can aid in our understanding of the ways in which important minerals are formed and processed in the subsurface, as well as the ways in which these minerals provide a suitable habitat for unique forms of life (Skinner and Catherine 1997). Microbes can affect the availability and mobility of minerals in the subsurface, such as metals and other material of economic importance. In addition, these microbes often utilize unique metabolic pathways and enzymes that could be of use in bio-mining or in pharmaceutical development (Nicolaidis 1987).

Studying subsurface life in impact craters could yield clues about the origins and evolution of life on our planet. Understanding the effects that an impact can have on these communities is essential in determining their ability to recover from or survive such events and the overall stability of communities during periods of high bombardment rates, as was likely the case on the early Earth during life's first appearance.

1.2.4 Geomicrobiology and Impact Craters

Impact craters are rarely found on the surface of the Earth today due to geological processes like plate tectonics, volcanism, erosion and weathering. These natural processes serve to erase the evidence of impacts over time as part of the normal cycling of rocks at the Earth's surface. In contrast, the surface of a celestial body like the Moon has experienced little or no re-surfacing since early in its formation, which is why we are able

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to easily spot numerous surface craters. In fact, examining craters on locations like the Moon is how we are able to estimate the number of impactors that have collided with Earth in the past, and to define historic periods in which our planet was experiencing more frequent impacts (Glikson 2001; Gomes *et al.* 2005; Gronstal *et al.* 2007). Even though impact structures are rarely found on the surface of the Earth today, our planet has experienced a large number of impacts since its formation.

Impacts on Earth have played an important role in the evolution and continued development of life. Objects like asteroids and comets are thought to be associated with many mass extinction events and periods of severe climate change in Earth's history (Claeys *et al.* 1992; Hut *et al.* 1987; Kerr AC 1998; Raup 1986). Many scientists believe that impacts could have played a role in the origins of life on the early Earth by delivering important precursor molecules for the first living cells (Gronstal *et al.*, *In Press* (b); Maher and Stevenson 1988).

It has traditionally been assumed that impact events generate sterile sections of subsurface sediments due to the thermal impulse generated by impact. Some researchers believe that early in the Earth's history, the frequency and size of impacts would have been sufficient to flash-heat the surface of the planet and vaporize the oceans (Amy and Haldeman 1997, p. 216). Life as we know it cannot exist above 120°C, and impacts can generate temperatures well in excess of this, and that can persist for extended periods of time. Based on the impact history of the moon, scientists determined that life could not have persisted in the face of the sterilisation-effects of impacts until the frequency of impacts was reduced some 3.8 billion years ago (Sleep *et al.* 1989). However, we now know that rocks from this period already contain highly evolved microorganisms, suggesting that some refuge for life allowed organisms to exist and evolve on Earth in spite of impact action (Amy and Haldeman 1997, p 216; Schopf 1983). In the deep subsurface, microorganisms may have been protected from the sterilizing affects of impact at the surface (Amy and Haldeman 1997, p 216). The earliest habitable environment on Earth

was likely in the deep subsurface, shallow enough to be above the line where geothermal heat is in excess of 120°C, but deep enough to be out of reach from impacts (Amy and Haldeman 1997, p. 216).

Impact craters can also affect communities of organisms by dramatically altering the local ecology in such a way as to create unique microenvironments for life to re-colonize after the initially devastating impact event. Many craters on Earth that have been identified were initially discovered and characterized based on the way in which vegetation re-grew in the area of impact or other ecological signs that some form of disturbance had occurred (Cockell and Lee 2002). There is also some indication that impact craters themselves could present novel environments for endolithic microorganisms at the surface, or even below the surface (Cockell and Lee 2002). Impact craters may present unique environments for subsurface organisms that are often very different from neighbouring subsurface environments found outside of the impact site (Cockell and Lee 2002). A large-scale impact event, such as the Chesapeake impact, causes a massive disruption of the natural environment at the point of impact, which is then followed by recovery. During this recovery, new organisms move in and take advantage of the microenvironments created by the impact and the lack of competition from other organisms in the region that have been wiped out by the impact. Impacts have in fact been referred to as 'biologically resetting' events (Cockell and Lee 2002), because the intense temperatures and pressures associated with the event causes a momentary sterilization of the local environment. This process is not dissimilar to the recovery of ecosystems after events like volcanic eruptions or large-scale fires; and as with these events, impact sites then form primary succession habitats once organisms begin the re-colonization process (Cockell and Lee 2002).

Today, one hundred and fifty impact craters have been recorded on Earth in a multitude of environments ranging from tropical forests to arctic tundra. The amount of variation in subsurface environments in these locations is poorly understood. One feature unique to all of these impact-generated environments in terms of microbial communities is

the presence of impact-shocked rocks. The force of impact can generate rocks that are highly compressed and with relatively little pore space for water and microorganisms. Additionally, impacts can also generate significant fracturing of rocks and increase the potential pore space available for microbial habitation. Other common elements of these impact environments are the presence of pore waters in rocks and crater material that may be left over from the impact event itself, and the presence of impact-derived hydrothermal systems caused by the massive release of kinetic energy during impact.

Studying the microbial communities present in crater environments can help us understand the environmental limits for life, and the ability of subsurface ecosystems to recover after potentially devastating environmental events. These sites also provide an important analogue for how life could survive in other subsurface locations in the solar system. If life were once present on Mars, deep below the planet's harsh surface, could communities have persisted through periods of heavy impact rates? Similarly, could the subsurface of the early Earth have provided a safe haven for microbial life while the surface was pummelled by impactors during the period of high impact activity known as the 'Late Heavy Bombardment'?

1.2.5 Relevance of the CBIS

Prior to the 2005 drilling of the CBIS, no evidence of microbiological communities present in the breccias[4] of the impact structure had been produced (Larsen and Crossey 2004). Mineralogical and chemical data obtained from earlier drill cores and data concerning the levels of organic carbon present in the structure suggested that microbes could be present (Larsen and Crossey 2004). The intriguing data from drilling efforts prior to that of 2005 was what informed the selection of microbiology and molecular biology studies as a key component of the 2005 ICDP-USGS deep drilling of the CBIS. The previous work formed the basis for studies performed by the team of biologists at the CBIS site. Numerous

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[4] Breccia is a rock containing angular fragments in a matrix. Breccias in the CBIS are formed by impact alteration.

proposals for biological studies were put forward (Larsen and Crossey 2004), and a significant portion of the 2005 project was devoted to geobiology. The 2005 corehole was the first to implement a full suite of contamination assessment methods to ensure that core samples retrieved from the subsurface were suitable for use in these studies (See **Chapter 2**), providing biologists with the first look into the subsurface ecology of an impact crater.

As discussed, the CBIS represents a unique habitat for subsurface life. Highly saline waters and the potential presence of impact-generated hydrothermal environments were some of the conditions anticipated prior to drilling. The extent of thermal heating experienced in the central region of the CBIS is unknown. Estimates by Sanford (2003) prior to the 2005 project placed the temperatures as high as 450°C in the resurge breccias even 10,000 years after the impact event. Indications of thermal heating outside of the central region have also been discussed (Larsen and Crossey 2004). Previous work on other marine craters, such as the Lockne crater in Sweden, indicate that the marine environment may act to dampen the amount of thermal heating in marine impacts compared to similarly sized events on land. The Lockne crater experienced a relatively short and low temperature hydrothermal system as a result of the crater-forming impact (Sturkell *et al.* 1998). This is not necessarily the case for the CBIS however, because it was formed in relatively shallow water and the impact may have excavated enough material to reach the crystalline basement rock that sits below the marine seafloor sediments. If the impact was indeed powerful enough to target the crystalline basement rock beneath marine sediments, heating caused by the thermal impulse could have been significantly higher due to the interaction with the hard, compact rock of the basement as compared to sediments (Rybach *et al.* 2004; Vosteen and Schellschmidt 2003). If this thermal pulse was great enough, its effects could have persisted long after the resurge waters and other materials filled the crater, and as the structure was subsequently covered with insulating sediment (Malinconico *et al.* 2008). Data returned in 2000 from the USGS-NASA Langley corehole at Hampton, Virginia, showed that the thermal disturbance in this outer region of the CBIS

lasted for 0.1 to 1 million years, but that the heating experienced was likely no higher than 100°C to120°C (Horton *et al.* 2005b). This is potentially below the sterilizing temperature for some known microorganisms on Earth today. In the central region of the CBIS, where crystalline basement rock was affected, these temperatures could be far greater. Breccias retrieved from the 823-meter-deep test hole drilled in Cape Charles, VA, (**Figure 6**) indicate that post-shock temperatures could have reached ≤550°C (Horton *et al.* 2006).

1.2.6 Studies reported here

Prior to the 2005 ICDP-USGS deep drilling of the CBIS, the environments for microbial life in the CBIS were unknown. My work on this project focused on answering the questions of whether or not the breccias present inside the impact structure were capable of supporting life, whether or not indigenous communities were present today and, if present, has the potential isolation of CBIS microbial communities produced microbial species that are distinct from currently known organisms native to the terrestrial subsurface. To answer these questions, numerous routes of evidence were explored. Total cell numbers present in CBIS core samples were enumerated by use of direct cell counts (**Chapter 3**). Organisms were cultured from CBIS core sediments and maintained under laboratory conditions (**Chapter 4**). Individual isolate organisms were obtained from the microbial communities retrieved from depth in the CBIS core, and these isolates were characterized to determine species type and physiological characteristics (**Chapter 5**). Molecular biology studies were also performed on CBIS cultures to identify potential species present in enrichment cultures. Molecular studies were also used to directly extract and analyze DNA present in CBIS core material (**Chapter 4**).

Together, these studies provide the first characterization of the subsurface microbial population associated with an impact crater, and a view into a potentially economically important community of microorganisms that inhabit groundwater reserves for a large and growing human population. This work, for the first time, also provides insight into how subsurface microbial communities respond to the convulsive geological

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event of bolide impacts. The recovery of communities following potentially sterilizing forces, and the generation of new habitats through fracturing of rock is vital in understanding how early life on Earth could have survived periods of heavy bombardment. This also has applications in understanding the affects of impact on habitability in the subsurface of other celestial bodies, like ancient Mars.

1.3 SUMMARY

The Chesapeake Bay Impact Structure was not fully identified until late in the 20th century, although its impact on the environment local to the Chesapeake Bay had been documented by human inhabitants for nearly a century. The CBIS has a profound effect on local economies, particularly due to its effects on local groundwater reserves.

In addition to its importance to the local economy, the CBIS can also yield important information about the habitats for life that can be generated by impact events. The CBIS contains unique subsurface conditions, including ancient saline and potentially geothermally heated water. This environment may harbour equally unique types of subsurface microorganisms that have been spatially isolated from other communities outside of the crater for significant periods of time. The microbial communities of the CBIS may have metabolic traits that could be useful in other fields, such as medicine and biomining.

As part of the 2005 ICDP-USGS deep drilling of the Chesapeake Bay Impact Structure, I have been conducting geobiology studies in microbiology and molecular biology to characterize the subsurface microbial community present at depth in the CBIS. This body of work is presented here, and provides the first ever account of how microbial communities persist in the deep subsurface following a large-scale geological convulsion event. This work provides the first outline for how subsurface habitats recover, and the new types of microenvironments that are formed and in which microbial communities can gain a foothold for growth. Data returned by colleagues on the 2005 drilling project concerning the geology and chemical properties of the drill core was used to interpret the

results of geobiology studies. These lines of information are cited as external to my work, and cases in which I had no participation in developing or deriving the data are indicated.

Chapter 2: Obtaining Biological Samples from Cores of the CBIS

2.1 ABSTRACT

The difficulty of obtaining biological samples from deep subsurface environments is the primary reason that this important microbial habitat remains relatively unknown. The primary methods for collecting samples are by retrieving materials from deep mines (or caves) and through drilling. Each of these methods pose problems in terms of acquiring representative samples of the subsurface that are free from contamination introduced during the sampling procedure.

Unique sets of core subsamples were collected during the 2005 ICDP-USGS deep drilling of the Chesapeake Bay Impact Structure (CBIS) for both enumeration and culturing/molecular studies. Five methods for monitoring contamination during the collection of enumeration samples were implemented. Fluorescent microspheres were added to the core collection barrel during collection to mimic the ability of microorganisms to enter cores through fractures in the core material. A chemical tracer (Halon 1211) was infused into the drilling mud during drilling to trace the intrusion of mud into cores. Dissolved organic carbon (DOC) contained in porewater samples drawn from cores was characterized using excitation-emission matrix (EEM). Distinct DOC signatures for drilling mud and cores were compared in order to identify drilling mud contamination in core porewater. Polar organic compounds fore porewaters were also characterized and compared to unique spectra obtained for the polar organic compounds in drilling muds in order to identify potential drilling mud intrusion. Finally, 16S rDNA clone libraries were constructed from drilling muds to identify a list of potential contaminants that were then compared to cultures obtained from CBIS samples. Based on the results of these five methods, core samples were categorized according to the likelihood of contamination. Twenty-two of the fifty core samples collected for microbiological culture and culture-

independent analysis were free of contamination by all methods. The use of these five

sample assessments provides a comprehensive method for tracking both particulate and

dissolved contaminants introduced during the drilling procedure, and could be applied to

sample collection from other environments with low biomass.

2.2 INTRODUCTION

The deep subsurface of Earth is a microbial habitat that remains relatively unexplored and

uncharacterized today. This is primarily due to the fact that acquiring samples from the

deep subsurface can be extremely difficult, time consuming and costly. The primary ways

in which the deep subsurface can be accessed are through existing mines (and caves) (e.g.

Wanger *et al.* 2008), or through deep drilling. Both of these methods can provide access to

great depths beneath the surface.

Deep drilling requires the use of equipment and methods that are not necessarily

suitable for retrieving samples for biological studies. Drilling processes were not

developed with biologists in mind, and while cores can be acquired from great depths,

drilling operations used to access these depths carry with them an inherent danger of

introducing non-indigenous microorganisms into samples as the drill bit advances, while

the corehole is flushed with fluids, and when the core is extracted from the ground (Smith

et al., 2000a, 2000b; Smith and D'Hondt, 2006). This danger of contamination is

compounded by the fact that the microbial load in deep subsurface environments tends to

be extremely low relative to surface environments. Introduction of even a small number of

non-native species can dramatically affect the results of any analysis performed on the

obtained samples. Contamination can cause overestimation of total cell numbers and

biomass in cores. Non-native species can be grown in cell cultures from cores alongside

indigenous organisms and falsely identified as members of the deep biosphere community.

DNA from contaminant species can also be extracted from samples and amplified, again

misrepresenting the true population of organisms present in the subsurface (Smith and

D'Hondt, 2006).

As interest in the deep subsurface biosphere developed in the latter half of the 20th century, the potential for contamination when drilling with standard techniques was quickly recognized. Studies of the deep subsurface biosphere have previously employed a range of techniques in order to monitor and assess potential contamination from the aforementioned sources during drilling. Many of these methods have been tested during drilling operations with and without circulating fluids. In 1985, the U.S. Department of Energy initiated its "Microbiology of the Deep Subsurface" program, and field studies began in 1986 (Fliermans and Balkwill, 1989; Lehman et al., 1995). The initial study sites tested as part of this initiative were three boreholes located at the Savannah River plant in Aiken, South Carolina. In this study, core sediments were separated from the circulating drilling fluids by using sterilized Plexiglas insert tubes during collection (Fliermans and Balkwill, 1989). Once the cores were returned to the surface, the outer two-thirds of their exterior were pared away to remove any contamination present in exposed surfaces of the core (Balkwill et al. 1989; Fliermans and Balkwill, 1989; Phelps et al. 1989). This was done because researchers assumed that material near the exterior of the core was more likely to be contaminated than interior sections during drilling and sample handling (Balkwill et al. 1989; Fliermans and Balkwill, 1989; Phelps et al. 1989). Although sterilizing equipment and removing the exterior of cores is still common practice (i.e. Reed et al. 2002; Smith et al. 2000b), these methods alone may not be adequate for ensuring the integrity of samples.

Microbial contaminants can enter into the interior of cores through microscopic cracks and fissures (Griffin et al., 1997; Smith et al. 2000a, 2000b). This is particularly a problem during drilling projects where the corehole is flushed with drilling fluids at high pressures. Drilling fluids are circulated through the entire length of the hole, from the surface down to the bit and back. These fluids inherently expose the exterior of the core to a large number of potential contaminants. When fluids penetrate into the interior of cores through cracks and fissures, they can potentially transport microbes into the core as well.

Numerous physical and chemical tracers have been used in previous studies to track drilling fluid intrusion into cores (i.e. Chapelle and Lovley, 1990; D'hondt *et al.*, 2004; Harvey *et al.*, 1989; Kallmeyer *et al.*, 2006; Lehman *et al.*, 2004; Smith *et al.* 2000b).

Fluorescent dyes have been used to visually identify drilling mud inside cores (i.e. Russell *et al.*, 1992). However, fluorescent dyes can be sensitive to conditions such as pH and oxidizing agents. There are also concerns over the toxicity of fluorescent dyes, which has limited their use in deep drilling projects (Griffin *et al.*, 1997). Other studies have attempted to use drilling fluids themselves as a tracer. This is possible when the fluids contain water-soluble ionic tracers that are easily detected in core samples (i.e. Russell *et al.*, 1992). Similarly, chemicals indigenous to certain fluids, including barium, sulfate and ammonium, have also been used to trace fluid intrusion (Griffin *et al.* 1997). However, these chemicals can also be present *in situ* in sediment porewaters, or they can sometimes be altered by biological and chemical reactions, affecting the ability to successfully identify them.

The use of perfluorocarbon tracers (PFTs) to identify drilling fluids in core samples is now more common than ionic tracers (i.e. Colwell *et al.*, 1992; Colwell *et al.*, 2001; Haldeman *et al.*, 1994; Haldeman *et al.*, 1995; House *et al.*, 2003; Lehman *et al.*, 2001; Lehman *et al.*, 2004; McKinley and Colwell, 1996; Russell *et al.*, 1992; Senum *et al.*, 1990; Smith *et al.* 2000a, 2000b; Sørensen *et al.*, 2004). PFTs have a number of advantages over ionic tracers as they are non-toxic, can be emulsified in drilling fluids and, importantly, are detectable at extremely low concentrations by pyrolysis gas chromatography and electron- capture detection (Griffin *et al.*, 1997).

One of the most popular methods for detecting contamination today is the use of fluorescent microspheres that simulate the ability of microorganisms to enter cores through cracks and fractures (i.e. Chapelle and Lovley, 1990; Colwell *et al.*, 1992; Colwell *et al.*, 2001; Fredrickson *et al.*, 1995; House *et al.*, 2003; Juck *et al.*, 2005; Kallmeyer *et al.*, 2006; Kieft *et al.*, 1995; Lehman *et al.*, 2001; Lehman *et al.*, 2004; Reed *et al.*, 2002;

Smith et al., 2000a, 2000b). Fluorescent microspheres are of similar average size to indigenous microorganisms present in soils and sediments, and can be detected visually by use of epifluorescence microscopy.

Microorganisms themselves can also be introduced into drilling fluids to trace contamination if the selected microbes are easily identifiable after penetration into cores (i.e. Beeman and Suflita, 1989; Colwell et al., 1994; Juck et al., 2005). In the past, studies have also used fluorescently tagged microbes (Juck et al., 2005) or microbes that are known to be indigenous to drilling fluids (Beeman and Suflita, 1989). However, the intentional delivery of non-native microorganisms into subsurface environments has been limited due to regulatory and environmental issues (Griffin et al., 1997).

Other methods for monitoring contamination involve comparisons between the physiological profiles of microbial communities present in core samples and in drilling fluids (Griffin et al., 1997). This includes techniques like constructing phospholipid fatty acid (PLFA) profiles of communities (Haldeman et al. 1994; Lehman et al., 1995), or by comparing the utilization of carbon sources by cultures from fluids and cores (Lehman et al., 1995).

To monitor contamination during the 2005 International Continental Drilling Program-United States Geological Survey (ICDP-USGS) deep drilling of the Chesapeake Bay Impact Structure (CBIS), five methods of contamination assessment were used. A number of the selected methods have proven useful in previous studies, such as PFTs and fluorescent microspheres. To add to these more traditional methods, profiles of polar organic compounds (Ronstad and Sanford, In Press) from core samples and drilling fluids were also constructed in order to identify intrusion of drilling fluids into cores. Additionally, a technique for comparing dissolved organic carbon (DOC) profiles of drilling fluids and core porewater samples was performed by researchers at the USGS in Reston, VA, and Denver, CO, and was implemented to assess sample integrity for the first time in a drilling project. The results of polar organic compound and DOC studies were

kindly released for comparison with results of the more traditional contamination assessment methods by the respective researchers involved in these experiments. For further details on polar organic compounds in the CBIS, see Ronstad and Sandford (*In Press*). For further details on DOC analysis of porewaters in the CBIS, see Sanford *et al.* (*In Press*). Both of these articles are currently being prepared for publication in a Geological Society of America special issue focused on the CBIS project.

A collective paper concerning all results of contamination assessment for biological samples can also be found in this publication (Gronstal *et al.*, *In Press*). I directly participated in preparation and delivery of microspheres and PFTs onsite at the CBIS drill site, as well as analysis of microsphere and PFT samples while on a research visit at the USGS in Reston, VA. Sample analysis for microspheres and PFTs were also performed by members of the research team in Reston, VA, and I would like to sincerely thank Elizabeth Jones, Nicole M. von der Heyde, Michael Lowit and Julie Kirshtein and Mary Voytek for their help in completing this time-consuming and sometimes tedious work.

Results of the selected methods were gathered, and the complete data set concerning all means of potential contamination was used to categorize the recovered microbiological samples according to the likelihood of contamination with a high level confidence.

2.3 MATERIALS AND METHODS

2.3.1 Field Site

The Chesapeake Bay Impact Structure (CBIS) was formed roughly 35 million years ago when an impactor collided with the continental shelf off the coast of what is now the eastern United States (Koeberl *et al.*, 1996; also see **Chapter 1**). The structure was formed in a shallow marine setting and is the best preserved of its kind on Earth (Koeberl *et al.*, 1996; Poag et al., 2004; Powars and Bruce, 1999).

Deep drilling of the CBIS took place from September to December of 2005 (Gohn 2006a, 2006b, 2008), and reached a final composite depth of 1.76 km. Drilling was

performed roughly 7 km north of Cape Charles on the eastern shore of Virginia (N 37°19.3', W 75°58.54; **Figure 7**). The drilling site rests above the circular moat that surrounds the central uplift of the crater, and is roughly 9 km from the centre of the structure (**Figure 1**).
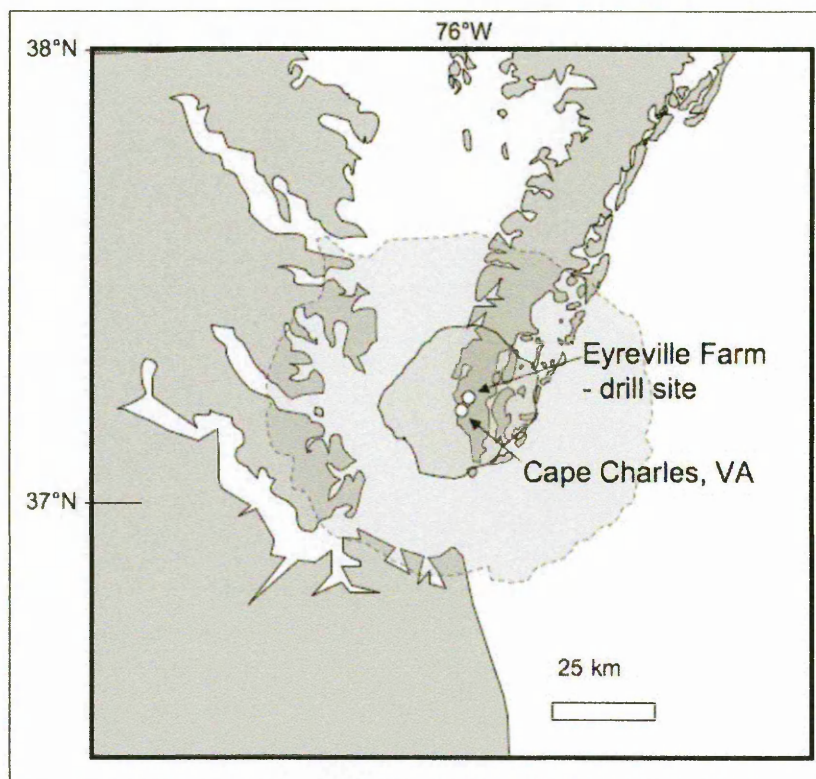
Figure 7 - The location of the Chesapeake Bay Impact Structure and ICDP-USGS drill site (Gohn et al., 2006a).

The Drilling Process

The hole of the 2005 ICDP-USGS deep drilling of the CBIS was bored by rotary drilling to a depth of roughly 128 m. During rotary drilling, no core was collected. Large-diameter steel casings were installed to a depth of 125 m. The first corehole from which samples were collected, the Eyreville A corehole, began at this depth and extended from 125 to 941 m. The original drilling was halted when the drill bit deviated from the original hole during reaming, and a second corehole (Eyreville B) was then drilled from 733 to 1,766 m (Gohn *et al.* 2006a).

A conventional core drilling technique was used to capture cores, by which a core barrel was placed at the bottom of a string of drilling rods. As the core barrel was rotated, cores were captured as the barrel advanced through the ground. During this process,

drilling fluid (aka drilling mud) is circulated through the borehole in order to cool the drill bit and flush cuttings to the surface along with the drilling fluid as it is pumped out of the hole. By this method, cores are retrieved when the entire string of drilling rods are removed from the corehole.

Drilling began on September 15, 2005, by the principal contract driller, Major Drilling America, using a CP-50 wireline coring rig. A PQ sampling system, with rods of 85.0 mm in diameter, was used to the depth of 591.0 m, where mud circulation was lost and the PQ (CHD-124) rods were trapped in the hole. Drilling was resumed using an HQ coring system (63.5 mm diameter) to a depth of 940.9 m when mud circulation was lost again. The HQ rods and bit were then drawn upward to a depth of 591.0 m. Mud circulation was repeatedly lost due to expanding and sliding red-clay section in the corehole and the bit wasn't returned to the depth of 940.9 m until October 20th, 2005. At this point, the bit had deviated from the original corehole at the depth of 737.6 m. This is the reason that duplicate cores were collected between 737.6 and 940.9 m. The corehole below the deviation point was then designated as the 'Eyreville B' corehole.

Coring with the HQ system continued to a depth of 1,100.9 m, at which point the HQ bit was deliberately stuck within a block of granite. This meant that the HQ casing could be used as a casing against the red-clays. An NQ (47.6 mm diameter) sampling system was then used to the final depth of 1,766.3 m (Gohn *et al.* 2006a).

A third core was obtained in April and may of 2006 using a USGS truck-mounted Mobile B-61 wireline coring rig. This provided sampling up the upper part of the post-impact sedimentary section using an HQ coring rig. However, this third section of core (Eyreville C) was not used as part of the studies contained in this thesis. The post-impact sedimentary section contains sediments that accumulated on top of the crater structure over the past 35 million years and are not expected to differ greatly from surrounding sediment structures on the Virginia coastal plain. As these sediments do not contain materials that were targeted by the impact event, the microbial communities present were not thought to

be of interest in determining the affects of impact cratering on subsurface microbial communities. Table 1 provides an outline of the core sections obtained from the CBIS and the coring systems used for retrieving the cores.

Table 1 - Cored section in the Eyreville coreholes (data from Gohn *et al.* 2006b). *The Eyreville C core was not sampled as part of this study.

Core Acquired	Depth (meters)	Coring System
Eyreville A	125.6 to 591.0 m	PQ core (85.0 mm diameter)
	591.0 to 940.9 m	HQ core (63.5 mm diameter)
Eyreville B	737.6 to 1,100.9 m	HQ core (63.5 mm diameter)
Eyreville C*	0 to 140.2 m	HQ core (63.5 mm diameter)

Samples for microbiological studies of the CBIS were collected and processed for storage and shipment on site according to procedures specific to the type of subsequent analysis to be performed. A total of 50 core subsamples were collected for culturing and culture-independent analysis (See **Chapter 4** for additional information on collection procedures). Two hundred and sixteen subsamples of cores (two subsamples per retrieved core) were collected for enumeration studies (See **Chapter 3** for additional information on collection procedures).

Care was taken during sub-sampling of retrieved cores to avoid regions that showed obvious breaches, such as cracks and fissures in the core exterior. Selecting appropriate sections of the core for sub-sampling was the first step in acquiring scientifically useful samples. The following contamination assessment methods were then implemented during collection of samples to be analyzed for microbiology and molecular biology throughout the length of the core.

2.3.2 Microspheres

Particulate tracers such as fluorescent microspheres are one of the most common methods for monitoring contamination in samples from the terrestrial subsurface and deep-sea sediments (see **Table 4**). Fluoresbrite Caboxylate Microspheres (Polysciences Inc. #15700)

were used as a particulate tracer during drilling of the CBIS. These microspheres are roughly 0.496 (± 0.014) μm in diameter and fluoresce bright green when observed under an epifluorescent microscope using a blue filter set (Zeiss filter set 09 or 10) (Smith *et al.*, 2000a, 2000b). Microspheres are similar in diameter to the average size of microorganisms expected to be indigenous to the subsurface environment of the CBIS and have been successfully implemented in many previous drilling programs (Harvey *et al.*, 1989; Smith *et al.*, 2000a, 2000b; Smith and D'Hondt, 2006).

Preparation and Delivery of Microspheres

Fluorescent microspheres were introduced into the core barrel during collection and retrieval of cores and were not present continually throughout the drilling process. Microspheres were only introduced during core collection in order to reduce costs, as only core samples being used for microbiology and molecular biology studies required monitoring for microbial contamination. The recommended concentration of microspheres is $\sim 10^{10}$ spheres/mL and was achieved by diluting 2 mL of the microsphere solution (3.78 x 10^{11} spheres/mL) into 40 mL of water and heat-sealing the final solution in a 15 cm plastic bag. Microspheres were packaged at the USGS in Reston, VA, prior to the initiation of the drilling program. I personally had no role in packaging and preparation as my participation in this project began at the drill site, where I ensured that pre-packaged microspheres were introduced into the core barrel at the appropriate times. Packaged microspheres were placed into the core barrel and held below the core catcher by a threaded collar. As the core entered the core barrel, friction ruptured the bag to release the microspheres along the length of the core section as the core was forced past the bag. Once the core was retrieved, the ruptured plastic package was examined in order to ensure that the microspheres were successfully delivered.

Collection of Samples

Immediately after cores were retrieved to the surface, a 6-10 cm section was selected before the core was washed for subsequent geological analysis. This selected subsection of

the core was immediately transferred to a UV-sterilised laminar-flow hood in the on-site microbiology laboratory. Whenever possible, the outmost rind of the core was removed – although at times the material composing the core was so rigid that exterior portions could not be cut away. All work spaces were thoroughly cleaned with ethanol between sampling, and all instruments were flame-sterilized and/or autoclaved. Latex or nitrile gloves were worn and core samples were handled on pre-autoclaved pieces of sterile foil. Subsamples from cores were collected using pre-autoclaved sterile spatulas. Sterile tongs were used to transfer core sections to fresh pieces of sterile foil for each step in the handling and sub-sampling process.

A sample of the drilling fluid was collected from the rind (i.e. the exposed exterior) and analyzed for the presence of microspheres in order to ensure that they had been successfully delivered. The exterior surface of the core was then quickly flamed in order to remove any perfluorocarbon chemical tracers (PFT). Additional subsamples were collected by either subcoring[5] the interior of the core or by cracking open the core with a flame-sterilized hammer and chisel. The method used for exposing interior sections of the core depended on the composition of the core materials and the ease with which they could be subcored. Cores were split apart with the hammer and chisel rather than cutting them in order to avoid transferring microspheres, PFTs or contaminants from the exterior to the interior of the core by dragging them through with a knife blade.

Analysis of Samples
Three samples for microsphere analysis were taken from each core. These samples came from the exterior, within the first centimetre beneath the core surface, and from the interior of the core. The sample of drilling fluid collected from the exterior surface of the core was first analyzed to ensure that the microspheres were successfully delivered during retrieval of the core.

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[5] Subcoring was performed by cracking open the core to expose fresh material, and then driving a metal subcorer into the core lengthwise to remove a smaller core section from the interior of the original section retrieved from the borehole.

The analysis procedure for microspheres was based on similar studies conducted by the Ocean Drilling Program on seafloor sediments (Smith *et al.* 2000a, 2000b). In short, core subsamples from each region of the core (exterior, just below the exterior and the interior) were filtered by gentle suction onto black polycarbonate filters and placed on clean glass sides for viewing. Core samples were also collected from intervals where microspheres were not introduced in order to provide a control (i.e. Smith *et al.* 2000b; Reed *et al.* 2002). Microsphere analysis was performed on samples using epifluorescence microscopy at the U.S. Geological Survey in Reston, VA. Samples were viewed using a Zeiss Axioskop 40 microscope with the Zeiss filter set 10 (excitation 445-450 nm, emission long pass 515 nm).

2.3.3 Perfluorocarbon Chemical Tracers (Halon 1211 Gas)

The introduction of microspheres can provide some indication of how cell-sized particles can enter into drill cores. However, microspheres do not behave exactly like cells. The interaction of microspheres with mineral particles can be very different than that of true microorganisms, and the range of sizes and shapes of microbial cells varies greatly. Additionally, microspheres provide an incomplete assessment of contamination because drilling fluids are able to penetrate through small pores that can exclude physical tracers. For these reasons, a chemical tracer was selected in addition to microspheres in order to monitor the intrusion of drilling fluids into core interiors.

Chemical tracers should ideally be non-toxic, chemically inert, not easily absorbed into core materials and must also be readily quantifiable at extremely low concentrations (McKinley and Colwell 1996). It is also important that the natural background of the selected chemical tracer is low. In other words, the chemical must not be naturally present in the materials being sampled, as any natural occurrence could be mask the signal of the desired tracer or cause a false positive result (McKinley and Colwell 1996). Perfluorocarbon tracers (PFTs) satisfy many of the se requirements and are one of the most common chemical tracers used in drilling studies (McKinley and Colwell 1996, Smith *et*

al., 2000a, 2000b; Harvey *et al.*, 1989, D'hondt *et al.*, 2004; Kallmeyer *et al.*, 2006). The solubility of these gases in aqueous solutions is sometimes limited and can cause issues with volatility. However, PFTs are inert and are easily detected in sample cores (McKinley and Colwell 1996, Smith *et al.*, 2000a, 2000b; Harvey *et al.*, 1989, D'hondt *et al.*, 2004; Kallmeyer *et al.*, 2006). PFTs have been shown to be detectable with four orders of magnitude greater sensitivity than fluorescent dyes in some previous studies (Senum *et al.*, 1990).

PFTs were introduced into drilling mud by emulsifying the perfluorocarbon into water and continually introducing the PFT solution into the drill mud stream. PFTs travelled with the drilling mud and in any case where drilling mud penetrated into cores through pore spaces, PFTs were carried along as well.

PFTs were able to penetrate through pores that were small enough to exclude microspheres as well as most microorganisms expected to be present in drilling fluids. For this reason, PFTs are an indicator of core contamination by fluid, but not necessarily biological contamination. PFTs must therefore be used in conjunction with additional contamination assessment methods, such as fluorescent microspheres, which are more specific to biological contamination.

There are a number of important procedural considerations that must be made when using PFT tracers. Most PFTs have a low solubility and emulsifying the tracer in drilling fluid is required for successful delivery. Additionally, because PFT tracers are volatile, care must be taken when handling samples to prevent significant loss of PFTs to the atmosphere. PFTs can also be spread between samples on clothing or unclean lab spaces, and care must be taken to avoid this form of cross contamination, which does not originate from actual drilling processes. Transfer of PFTs in this study was not a major concern because the specific properties of the chosen PFT (Halon 1211). Temporal spacing between sampling times were significant (on the order of hours), meaning that PFTs present on surfaces would have more than ample time to dissipate into the atmosphere.

The chosen PFT for the 2005 drilling of the CBIS was Halon 1211 (bromochlorodifluoromethane). A pressurized stream of 1% Halon 1211 in N_2 was injected directly into the drilling fluid with a flow rate of 10 cc/minute, producing a final concentration of approximately 1ppm Halon-1211. Drilling fluids were pumped through the corehole at a flow rate of 18-23 gpm.

All of the gas mixture was dissolved into the drilling fluid at pressure, which was circulated to depth within the corehole and back to the land surface. Samples of the return-flow drilling fluid were collected, sealed in bottles and returned to the lab at the USGS in Reston, VA, to verify the concentration of Halon 1211 and ensure a successful delivery of the PFT tracer.

Collection of Samples

Samples were collected from the exterior and interior of core sections by the same method described above for fluorescent microspheres. Samples of the drilling fluid that coated the surface of cores were collected and stored in sterile, pre-weighed headspace vials that were sealed with gas-tight stoppers. These samples were collected, stored and sealed as quickly as possible to reduce the loss of Halon 1211 to the atmosphere through volatilization. These samples were tested to ensure successful delivery of Halon 1211 at depth in the corehole.

If possible, drilling mud rinds were then removed. Halon 1211 was then eliminated from the surface by passing the core samples through a flame until it was dry. This reduces the risk of transferring PFT tracer to the interior during handling. Drying the exterior surface cores has been shown to be the most effective method for removing PFT (Smith *et al.* 2000a, 2000b). However, care must taken to avoid overheating the samples as heating and desiccation could affect further microbiological analysis by damaging otherwise cultureable cells.

Core sections were then transferred to a fresh piece of sterile foil using sterile tongs. The exterior of the core was pared by use of a sterile hammer and chisel. All

instruments used in handling the core were passed through a flame at each step in the process to remove any PFT and sterilize their surfaces (Smith *et al.*, 2000a, 2000b). Splitting the core open exposed interior sections. Samples were collected from near the surface of the core and from the centre of the core. These samples were also transferred into sterile, pre-weighed headspace vials and sealed with gas tight stoppers. Again, core handling and sample collection was performed as quickly and with as little manipulation as possible to avoid loss of Halon 1211.

It was assumed that some of the Halon 1211 would be lost to the atmosphere during the brief period when the cores was extracted and sampled at the land surface. However, even if 99% of Halon 1211 was lost, the quantity emulsified in drilling fluids was sufficiently high that sample vials would still contain concentrations on the order of 10 ppb. This is more than 1000 times higher than the detection limit for the electron capture detector (ECD) used to analyse samples

Analysis of Samples
As with the fluorescent microspheres, procedure for PFT analysis was adapted from similar studies on deep ocean cores collected by the ODP (Smith *et al.* 2000a, 2000b). Samples collected from the core were transferred to the USGS in Reston, VA, and analysed using Gas chromatography (GC) in the Reston Trace Gas and Chlorofluorocarbon Laboratory (http://water.usgs.gov/lab/). The majority of these samples were analysed by Elizabeth Jones at the USGS in Reston, VA.

2.3.4 Clone Library of Drilling Mud
Clone libraries were constructed from drilling mud as an additional contamination assessment after the initial screening of CBIS samples was performed with the aforementioned methods. Information from16S rDNA clone libraries was used to characterize the microbial communities present in drilling fluids and, ultimately, to produce a list of potential contaminants. This list was then compared to microorganisms present in cultures obtained from CBIS core sediments. If cultures produced a potential

contaminant from the list, the contaminant organism was then eliminated from further culturing and molecular studies.

DNA extraction from drilling mud was performed using the ultraclean Soil DNA isolation kit (MoBio, Inc., Carlsbad, CA) during a research visit in the Voytek Microbiology Laboratories at the USGS in Reston, VA. Bacterial DNA was amplified from drilling-mud DNA using the polymerase chain reaction (PCR) in a Perkin Elmer Geneamp 2400 thermal cycler with bacterial 16S rDNA (46 forward and 519 reverse) primers (Brunk et al. 1996; Lane 1991). The conditions for 16S rDNA PCR (30 cycles) were: denaturing at 94 °C (30s), annealing at 56 °C (30s), and extension at 72 °C (1m). Cloning and sequencing the 16S rDNA was used to characterize the microbial community contained in drilling mud. The amplicons obtained were purified using the Wizard PCR purification kit (Promega, Madison, WI) and cloned using the TA cloning kit according to manufacturer's instructions (Invitrogen, San Diego, CA). Successful colonies resulting from cloning were then selected and 16S rRNA gene clone fragments were recovered by PCR with vector primers M13f and M13R. Products from PCR were then re-amplified using 46f and 519r primers. All PCR amplicons were digested with restriction enzymes (6 μL of PCR product with 2.5U each of MspI and HinPI) according to manufacturer's instructions (Promega, Madison, WI). Restriction fragments were analyzed by size separation on a 3.5% Metaphor™ (Cambrex, Rockland, ME) agarose gel, restriction fragment length polymorphism (RFLP) patterns were distinguished, and the frequency with which each pattern occurred was determined. Representative clones for each pattern were selected for sequencing. Amplicons to be sequenced were purified with the Wizard PCR purification system, and cycle sequencing was performed on both strands of the DNA product using big dye v3.1 (Applied Biosystems, Foster City, CA) and run on an ABI310 genetic analyzer. Sequences were edited and assembled using Autoassembler.

2.3.5 Laboratory Contaminants

To prevent the introduction of contaminant organisms into cultures in the laboratory, all work surfaces inside the gloved anaerobic workstation (miniMACS Anaerobic Workstation, Don Shitley Scientific Limited) were cleaned with ethanol after each use. Swabs from surfaces inside the anaerobic workstation were also used to inoculate growth media used for obtaining CBIS cultures. Any successful cultures obtained from these swabs were then sequenced in order to identify potential contaminants.

2.4 RESULTS

In total, fifty samples for culturing and genomic analysis were collected at depths throughout the CBIS core. Additionally, two hundred and sixteen samples (at least two samples from 98 different depths) were collected for direct cell enumeration studies throughout the length of the core. The four primary methods for contamination assessment discussed above (microspheres, PFTs, DOC analysis and clone libraries) were implemented in order to obtain core samples suitable for culturing and genomic studies. Because of this, use of these techniques was focused on depths where core samples for culture studies were collected. These fifty depths did not always overlap with the depths where core samples for enumeration studies were collected, meaning that not all samples for enumeration were monitored by all the techniques discussed.

With the successful technique developed by Rostad and Sanford (*In Press*) for monitoring drilling fluid intrusion by acid polar component analyses, data concerning contamination in additional depths throughout the CBIS core was made available. By examining the results provided by Rostad and Sanford (*In Press*), we were able to ensure that CBIS cores where enumeration samples were collected – but where the full suite of contamination assessments were not implemented - were free from contamination.

2.4.1 Culturing Samples

A total of fifty core samples were collected from CBIS cores for culturing and genomic analysis. Samples for contamination assessment were collected at all fifty of these depths

and were first analyzed for the presence of fluorescent microsphere and Halon 1211 at the USGS in Reston, VA (Figure X). Based on the results of this analysis, samples were binned into three categories.

1) <u>Uncontaminated</u>: Samples were placed in the 'uncontaminated' bin if microspheres and Halon 1211 showed no sign of contamination. Additionally, if only one test for microspheres or Halon 1211 showed no contamination while the other was either non conclusive or a sample was not collected, and both DOC and Polar Components showed no contamination, samples were included in this group. This category included 22 of the 50 total samples.

2) <u>Likely Not Contaminated</u>: In some cases, one technique showed no indication of contamination while other techniques were inconclusive or samples were not collected. Samples were binned as 'likely not contaminated' when at least one method showed no contamination, and the results from further contamination assessments were used to determine the usefulness of these samples. Additionally, samples that were inconclusive or not collected with microspheres, Halon 1211, DOC and Polar Components were placed in this category for further examination using additional data from sources such as the clone libraries. This category included 22 of the total 50 samples.

3) <u>Likely Contaminated</u>: Samples were binned as 'likely contaminated' when either microspheres or Halon 1211 indicated contamination. Six of the total 50 samples showed potential contamination by one of the two methods. In some cases, when these samples were from regions of particular interest within the core, further examination of contamination by other methods was used to determine whether or not these samples could be used to provide any useful data.

4) <u>Definitely Contaminated</u>: Samples were to be placed in this bin when all methods indicated contamination as well as additional data from clone libraries. These
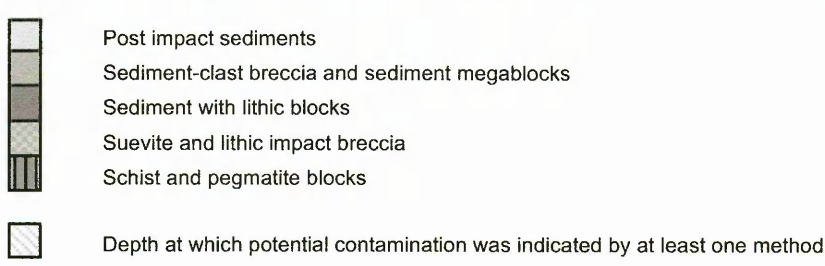
samples were those that could be classified as fully contaminated with high confidence.

Failure in deliveries of Halon 1211 or microspheres at certain depths was the primary reason for inconclusive results cited for Category 2 (above). When for instance, Halon 1211 was unsuccessfully delivered due to equipment failure, no Halon 1211 would be detected on the exterior or in the interior of core samples. Because no Halon 1211 was present, the absence of Halon 1211 inside the core did not indicate that the sample was uncontaminated, and instead the sample was marked inconclusive for that depth (**Table 2**). Similar problems arose with microspheres when sachets containing spheres did not successfully rupture inside the core barrel during core collection and retrieval. If microspheres were not successfully delivered, they of course could not be used as a proxy for microbial intrusion into the interior of cores.

Attempts to culture microorganisms from CBIS core samples were focused on the 22 core samples drawn from category 1. Additional samples from category 2 and 3 were re-evaluated for their potential use in regions of the core that appeared to be of particular interest based on chemical data obtained from the USGS. The results of the DOC analysis (Sanford *et al.*, *In Press*) and polar organic compounds (Ronstad and Sanford, *In Press*) were used to cross-check the likelihood of contamination in samples used in culturing and molecular biology studies (**Table 2**), as well as those used in enumeration studies.

Table 2 - Map of core segments collected for microbiology studies and corresponding regions that show contamination from either microspheres, PFT, DOC analysis or Acid Polar Component analysis. Data for Acid Polar components is derived from Ronstad and Sanford (In Press). 'E' indicates depths where samples were collected strictly for enumeration studies (see Cockell et al., In Press). Samples marked 'C' are those judged to be contaminated and those marked 'NC' are not contaminated. Samples marked 'rejected' are those that were not countable due to a failure of microsphere delivery or an insufficient concentration of PFT in drilling fluid during sampling. An 'x' indicates a depth at which samples were not collected for analysis. Samples collected for enumeration studies were not categorized along with samples for culturing, and are marked as 'N/A' in the column 'Category of Sample'. The depths and sample numbers where culturing studies were performed are highlighted in bold.

Sample Depth Meters	CORE	Core Samples	HALON 1211	Microspheres	DOC	Polar Components	Category of Sample
126.9		1	NC	x	NC	x	2
135.5		E	x	x	NC	NC	N/A
153,7		E	x	x	NC	NC	N/A
159.3		**2**	Rejected	x	NC	NC	2
180.6		E	x	x	NC	NC	N/A
204.2		E	x	x	NC	NC	N/A
225.2		E	x	x	NC	NC	N/A
241.5		3, 4	Rejected	x	x	NC	2
250.5		**5**	NC	NC	NC	x	1
276.2		E	x	x	NC	NC	N/A
360.2		6, 7	NC	Rejected	NC	NC	1
366.6		**8**	NC	Rejected	x	NC	2
410.9		E	x	x	NC	NC	N/A
435.6		9	NC	Rejected	NC	NC	1
441.7		**10**	NC	NC	x	x	1
447.0		11	Rejected	Rejected	x	NC	2
449.2- 449.4		**12**	Rejected	NC	NC	NC	1
459.3		E	x	x	NC	x	N/A
492.0		E	x	x	NC	x	N/A
500.8		**13**	NC	NC	x	x	1
510.6 - 510.7		**14**	NC	Rejected	NC	NC	1
524,2		E	x	x	NC	NC	N/A
544.2 - 544.3		**15**	C	NC	x	x	3
571.0 - 571.1		**16**	C	Rejected	NC	NC	3
583.0		E	x	x	NC	NC	N/A
588.1		**17**	NC	Rejected	x	x	2
614.5 - 614.7		**18**	Rejected	Rejected	NC	NC	2
627.1		E	x	x	NC	NC	N/A
643.4 - 643.6		**19**	Rejected	C	x	x	3
665.9		E	x	x	NC	NC	N/A
677.7 - 677.8		**20**	NC	Rejected	NC	NC	1
702.5 - 702.6		**21**	NC	NC	NC	x	1
744.5 - 744.6		**22**	C	NC	x	x	3
770.7 - 770.8		**23**	NC	NC	x	x	1
787.3 - 787.4		**24**	Rejected	Rejected	NC	x	2
808.1		E	x	x	NC	x	N/A

Depth		Sample					Score
821.0 - 821.1		25	C	C	X	X	3
842.0 - 842.2		28, 29	NC	Rejected	NC	NC	1
844.5 - 844.7		26	NC	Rejected	X	NC	2
862.0		E	x	x	NC	x	N/A
889.2		E	x	x	NC	NC	N/A
899.1 - 899.2		27	NC	NC	X	X	1
900.9 - 901.0		30	NC	NC	NC	x	1
941.9 – 942.0		31	NC	Rejected	NC	NC	1
958.3		E	x	x	NC	x	N/A
970.3 - 970.4		32	NC	NC	x	x	1
980.9		E	x	x	NC	x	N/A
997.7 - 997.8		33	NC	Rejected	x	x	2
1020.1 - 1020.3		34	NC	NC	x	x	1
1040.1		E	x	x	NC	NC	N/A
1047.7 - 1047.8		35	NC	NC	NC	x	1
1085.4 - 1085.5		36	NC	NC	x	x	1
1094.2		E	x	x	NC	x	N/A
		Granite Megablock					
1391.8 - 1392.0		37	Rejected	NC	NC	x	2
1419.0 - 1419.1		38	Rejected	Rejected	x	x	2
1447.4 - 1447.5		39	Rejected	C	x	x	3
1473.2 - 1473.3		40	Rejected	Rejected	x	x	2
1509.3 - 1509.4		41	Rejected	NC	NC	NC	1
1537.3 - 1537.4		42	Rejected	NC	x	x	2
1562.4		43	Rejected	Rejected	NC	NC	2
1567.0 - 1567.1		44	Rejected	NC	x	NC	2
1568.6 - 1568.5		45	Rejected	NC	NC	NC	1
1587.4		E	x	x	NC	NC	N/A
1608.7 - 1608.8		46	Rejected	Rejected	x	x	2
1642.5		47	Rejected	Rejected	x	x	2
1677.3 - 1677.5		48	Rejected	Rejected	NC	x	2
1740.4 - 1740.5		49	Rejected	Rejected	NC	x	2
1752.5 - 1752.6		50	Rejected	Rejected	x	x	2

Legend:

- Post impact sediments
- Sediment-clast breccia and sediment megablocks
- Sediment with lithic blocks
- Suevite and lithic impact breccia
- Schist and pegmatite blocks

- Depth at which potential contamination was indicated by at least one method

16S rDNA sequences from organisms that were successfully cultured from the 22

cores in category 1 and additional cores from category 2 were compared to those obtained

in the clone libraries drawn from the drilling mud. Cultured organisms were discounted

from study if they showed similarity to organisms identified in drilling-mud clone libraries.

Clone libraries of the various drilling muds used revealed that all muds contained potential

microbial contaminants. Clones obtained from drilling fluids tended to represent microbial

subphyla typical of soil, including *Clostridia* and *Citrobacter* (Janssen, 2006; Smit *et al.*,

2001) (**Table 3**). Potential contaminant organisms were then cross-referenced with

organisms identified in core samples either through molecular studies or through culturing

studies in the laboratory. None of the potential contaminants appeared in cultures obtained

from cores. Efforts to characterize these potentially novel organisms form the deep

subsurface are ongoing.

Table 3 - Sequences obtained by clone libraries from drilling muds used during drilling of the CBIS. Drilling muds not listed returned no successful clones. The closest identified sequence matches are in bold. Sequences were compared using the NCBI Genebank.

Drilling Mud Clone	Closest Identified Match	Similar Matches (Included Uncultured Clones)	Max Identity
Drilling Mud 1	**Clostridium**	AJ746506.1 Uncultured eubacterium	88%
		AB437976.1 Uncultured compost bacterium	88%
		EU551119.1 Uncultured Clostridia bacterium clone	88%
		AB125279.1 Clostridium straminisolvens	86%
Drilling Mud 2	**Clostridium**	AJ746506.1 Uncultured eubacterium	90%
		EU551119.1 Uncultured Clostridia bacterium	91%
		AB125279.1 Clostridium straminisolvens	88%
		CP000568.1 Clostridium thermocellum	87%
		AB377176.1 Clostridiaceae bacterium	89%
Drilling Mud 3	**Clostridium**	AJ746506.1 Uncultured eubacterium	90%
		EU551119.1 Uncultured Clostridia bacterium	91%
		AB125279.1 Clostridium straminisolvens	97%
		CP000568.1 Clostridium thermocellum	89%
		AB377176.1 Clostridiaceae bacterium	89%
Drilling Mud 5	**Clostridiales**	AF320297.1 Uncultured SAB reactor bacterium	95%
		EU551090.1 Uncultured Clostridia bacterium	91%
		EU283554.1 Uncultured Clostridiaceae bacterium	87%

		AF481208.1 Clostridiales oral clone MCE3_9 E1	86%
		EU464477.1 Uncultured bacterium	98%
		AF025370.1 Citrobacter amalonaticus	98%
Drilling Mud 6	**Citrobacter**	AJ415574.1 Citrobacter amalonaticus	98%
		AY379978.1 Salmonella sp. AHL 6	98%
		CP000880.1 Salmonella enterica: arizonae serovar	98%
		AF025371.1 Citrobacter farmeri	97%
		EU551119.1 Uncultured Clostridia bacterium	91%
		AJ746506.1 Uncultured eubacterium	91%
Drilling Mud 8	**Clostridium**	AB437976.1 Uncultured compost bacterium	90%
		Uncultured Clostridium sp.	97%
		AB125279.1 Clostridium straminisolvens	97%
		CP000568.1 Clostridium thermocellum	89%
		EU551090.1 Uncultured Clostridia bacterium	91%
		DQ206423.1 Uncultured low G+C Gram-pos bact	87%
Drilling Mud 9	**Clostridium**	EU283554.1 Uncultured Clostridiaceae bacterium	87%
		AF261803.1 Uncultured manure pit bacterium P316	87%
		AY959944.2 Clostridium alkalicellum	89%
		EU266899.1 Uncultured Bacteroidetes/Chlorobi group	97%
		AF445251.1 Swine manure pit bacterium PPC1	93%
Drilling Mud 10	**Proteiniphilum**	**EU377684.1 Proteiniphilum sp. enrichment**	97%
		DQ178248.1 Ruminobacillus xylanolyticum	95%
		AY570690.1 Petrimonas sulfuriphila strain BN3	93%

2.4.2 Enumeration Samples

Enumeration studies were begun immediately after samples from the CBIS cores were collected and before the results of contamination assessments became available. Total cell counts were performed on all samples from all depths for enumeration (see **Chapter 3**). Results of the contamination assessments were then used to eliminate depths that showed potential contamination.

The only contamination assessment that could be performed in real-time during enumeration studies was viewing of fluorescent microspheres. Because enumeration of cell numbers was performed using epifluorescence microscopy, microspheres could also be

viewed while cell counts were performed if the sample originated from depths where microsphere were used, providing an additional test for contamination assessment. No microspheres were viewed in any of the samples collected for enumeration.

Many of the enumeration samples were collected from similar depths as culture samples, and results of the contamination assessment for culture samples could then be used to eliminate contaminated enumeration samples. For enumeration samples from depths where these assessments were not available, results from acid polar component studies performed by Ronstad and Sanford (*In Press*) were used to confirm that uncontaminated samples had been successfully collected (**Table 2**). In total, only five depths from which enumeration samples were collected showed signs of potential contamination.

2.4.3 Samples from the Granite Megablock

Cores retrieved from the granite megablock at depths between 1,096 to 1,371 m were composed of hard, solid granite that would sometimes arrive at the surface with segments of smaller, fractured pieces. This material was impossible to subcore in the laboratory, and the fractures were inundated with drill mud. Because of these issues, microbiological core samples were not collected from this region of the corehole.

2.5 DISCUSSION

Assessing the level of contamination in samples from the deep subsurface is essential in obtaining samples that are useful for microbiology and molecular biology studies. Deep drilling processes carry with them an inherent danger of allowing intrusion of non-native microorganisms, and such organisms must be identified in order to ensure the accuracy of culturing, molecular and cell enumeration studies. These methods for studying the microbial ecology of the deep subsurface cannot be successfully performed if samples contain unidentified, non-native microorganisms.

Obtaining materials from the deep subsurface of Earth is an expensive endeavour, and because of this, samples are typically acquired in large-scale drilling projects that

include a consortium of scientists from a wide range of disciplines, including geology,

hydrology and microbiology. The high costs of drilling means that drilling equipment and

techniques used obtain samples have been developed for other commercial industries, such

as oil and mining. These techniques were not developed with the intention of returning

biologically useful samples. Developing interest in the microbial ecology of deep

subsurface environments on Earth has resulted in the development of numerous techniques

to ensure that samples collected through drilling projects remain free of contamination.

These techniques range from studying *in situ* characteristics of drilling fluids to

introducing chemical and particulate tracers into drilling fluids in order to trace their

passage through core materials.

Table 4 – Contamination Assessment Procedures for Biological Samples in Previous Drilling Projects.

Reference	Research Site	Method of Sampling	Maximum Depth	Contamination Control	Additional Notes
Balkwill *et al.* (1989) Fliermans and Balkwill (1989) Jiménez (1990) Phelps *et al.* (1989)	Savannah River site, Aiken, South Carolina (3 sites)	Drilling	265 m 216 m 213 m	Sterile plexiglass insert tubes sealed with Teflon to keep sediments separated from fluids Outside of cores pared away	None
Beeman and Suflita (1989)	Atlantic Coastal Plain	Drilling	~225 m ~425 m	Microbial tracer	Non-faecal coliforms present in surface soil were enriched in drilling fluid
Boivin-Jahns *et al.* (1996)	Mol, Belgium	Drilling	Horizontally drilled from inside a mine at 224 m	Equipment cleaned before use (high-pressure wash, formalin solution, ethanol solution) Outside of cores pared away	Core was horizontally drilled to a total length of 20 m
Chandler et al (1997)	South-central Washington State	Drilling (cable-tool drilling with no circulating drilling fluid or air)	187.7-188.4 m	Outside of cores pared away	Integrity of clone libraries from interior of cores was checked by comparing clones obtained from PCR templates and from blank extracts
Chapelle and Lovley (1990)	Atlantic Coastal Plain of South Carolina, sandy sediments	Drilling	~225 m ~425 m	Fluorescent microspheres Water-soluble ionic tracers	None

Reference	Site	Method	Depth	Tracer/Test	Notes
Christner et al (2000)	Glacial ice cores from China, Greenland, Bolivia, and Antarctica	Drilling	Shallow	Autoclaveable sample system used to extract samples from core interiors Exposure of surface to 95% ethanol (2 min) Culturing tests of core surface for bacterial endospores 16S rDNA amplification to test for DNA on exterior of cores	Ice cores drilled from glaciers
Colwell et al. (1992)	Test Area North (TAN) site at the Idaho National Engineering and Environmental Laboratory (INEEL)	Drilling	183 m	Fluorescent microspheres Perfluorocarbon Lithium bromide used as downhole tracer	Argon used as inert drilling fluid
Colwell et al. (1994)	Not Applicable	Drilling	Not Applicable	Microbial tracer	Cyanobacteria used as a particulate tracer Laboratory test using basalt cores and simulations
Colwell (1989)	Radioactive Waste Management Complex, Idaho National Engineering Laboratory, south central Idaho	Drilling	~ 70 m	Surface pared away using a sterile spatula and interior subcored using a sterile syringe	Air-rotary drilling, no use of drilling fluids
Colwell et al. (2001)	Test Area North (TAN) site at the Idaho National Engineering and Environmental Laboratory (INEEL)	Drilling	~ 63-134 m	Fluorescent microspheres Perfluorocarbon tracers Community profiles of drilling muds and core samples	PFTs were used only below 95.7 m due to mechanical malfunction Microbial communities of mud and cores were compared by carbon source utilization tests Air-water drilling fluid disinfected with bleach
Fredrickson et al. (1995)	US Dept of Energy Hanford Site; South-central Washington State	Drilling	212 m (two separate sites)	Fluorescent microspheres (dry tracer mix, used in only one of two drill sites)	Cable tool drilling method without the use of drilling fluids
Haldeman et al. (1994)	400 m deep tunnel system at Rainier Mesa, Nevada Test Site	Mining	400m deep tunnel system	Samples aseptically chipped from fresh rock faces	Alpine miner used to obtain mined rock for sampling
Haldeman et al (1995)	400 m deep tunnel system at Rainier Mesa, Nevada Test Site	Drilling (perpendicular drilling within a tunnel system of 400m) Mining	400m deep tunnel system	Solute tracer (Lithium bromide)	A portable corer was used for perpendicular drilling Alpine miner used to obtain mined rock for sampling
House et al. (2003)	ODP Leg 201, Peru Basin (2 sites)	Drilling	275 mbsf* ~450 mbsf*	Fluorescent microspheres Perfluorocarbon tracers	None

Reference	Site	Method	Depth	Tracer/Control	Notes
Juck et al. (2005)	Ground ice cores, Canadian high arctic	Drilling	11.58 m (Deepest of three sample sites)	Fluorescent microspheres Pseudomonas strain expressing the green fluorescent protein	None
Kallmeyer et al. (2006)	Terrestrial site, North Island, New Zealand	Drilling	150 meters	Fluorescent microspheres	None
Kieft et al. (1995)	South-central Washington State	Drilling (cable tool, split-spoon sampling)	173.3 to 196.8 m	Fluorescent microspheres (yellow and red) Bromide solute tracer (added as KBr)	Also looked at Total Organic Carbon and inorganic Carbon, but not as a contamination control.
Lehman et al. (1995)	Texaco Thorn Hill #1 well in Westmoreland County, Virginia	Drilling	3113 m	Carbon source utilization PLFA profiles	Tests performed on cultures acquired from surface soils, groundwater, drilling fluids and deep subsurface cores (Triassic Sediments) Use of other contamination controls was prohibited
Lehman et al. (2001)	Acidic, Crystalline Rock Aquifer, Arizona	Drilling	120 m	Fluorescent microspheres Perfluorocarbon tracers	New drilling rods purchased and steam cleaned before use
Lehman et al. (2004)	Snake River Plain Aquifer, Idaho	Drilling (Reverse air-rotary coring)	134 m 126 m	Microspheres Perfluorocarbon tracers	Disinfected groundwater added to the air to remove cuttings and cool the drill bit
Malard et al (2005)	Lyon, France	Drilling (Air-actuated hammer)	26 m	None described	Studied heavy metals and nutrients in cores to determine the extent of sediment contamination during installation of ground water monitoring systems.
McKinley and Colwell (1996)	Texaco Thorn Hill #1 well in Westmoreland County, Virginia	Drilling	~172 m	Perfluorocarbon tracers	Mud-rotary and air-rotary drilling Two PFTs were used during air-rotary drilling in Coastal Plain Sediments
Reed et al. (2002)	Sea sediments of the Blake Ridge and Cascadia Margin	Drilling	+300 mbsf*	Fluorescent microspheres	Outside of cores was pared away Pressure and temperature core sampler system used between 230 and 309 mbsf*
Russell et al. (1992)	US Department of Energy field site	Drilling	N/A	Fluorescent microspheres Dyes Perfluorocarbon Water-soluble ionic tracer (Potassium bromide) Innate properties of drilling muds (Ammonium ions)	Sampling tools developed specifically for the project
Smith et al. (2000a, b)	Ocean Drilling Program (ODP) Leg 185, Pigafetta Basin, South China Sea	Drilling	191.2 mbsf* 445.2 mbsf* 426.7 mbsf*	Fluorescent Microspheres Perfluorocarbon Outside of hard sediments pared away	Outside of sediments were only pared away if the material was too hard to sample with a syringe

Sørensen *et al.* (2004)	ODP Leg 201, Peru Basin	Drilling	43 mbsf*	Perfluorocarbon	Samples frozen at -80°C Immediately after collection
Wilms *et al.* (2006)	Wadden Sea	Aluminum tubes driven into sediment using a vibrating unit	shallow	None stated	Sterile syringes were used to remove samples from the innermost part of cores

(*) meters below seafloor (mbsf)

Table 4 provides an overview of the types of contamination assessments that have been previously implemented in drilling projects of varying scales. The most common techniques cited in literature include fluorescent microsphere and PFT chemical tracers. These are considered to be two of the most robust techniques and have proven their effectiveness in many environments. Today, there is not yet a consensus in the research community as to which techniques are the most appropriate for contamination assessment during deep drilling projects. This is primarily because each of these techniques is best suited for identifying a specific type of contamination. Microspheres are designed to be of similar size to microorganisms that are expected to be present in drilling fluids and act as a proxy for microbial intrusion into cores. However, some microorganisms are known to be less than 0.5 μm in diameter, and it is possible that microspheres provide an incomplete assessment of potential contamination. PFTs trace penetration of soluble components in drilling fluids into sediments, and are able to intrude through pores and fractures that could be small enough to exclude microorganisms. To account for the shortfalls in the various methods, multiple techniques must be implemented in order to monitor the different ways in which contamination can enter cores. Additionally, implementation of multiple techniques means that there is always a backup in the event that one technique fails. The most prudent approach is therefore to implement as many assessment techniques as time and funding allows.

During the 2005 ICDP-USGS deep drilling of the CBIS, the first step in obtaining uncontaminated samples was performed during selection of core segments for experimental analysis. Cores were examined immediately upon retrieval and before handling and washing for geological studies. When selecting core sections, it was

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important to avoid sections that showed visible cracks or fragmentations. Solid sections of the core were selected that were sufficiently large to be cracked open and subsampled in the facilities onsite in the microbiology laboratory. Cores that showed obvious cracks or materials that were unconsolidated were not considered for study because drilling fluids and contaminant microbes would easily penetrate this fractured material. Therefore, the first step in contamination control was to carefully select the most solid section of continuous core from which to collect subsamples for microbiological and molecular analysis.

Beyond the physical examination of cores performed on site at the time of collection, a suite of four primary contamination assessments were implemented during the 2005 ICDP-USGS deep drilling of the CBIS to ensure that experimentally useful samples were obtained. A fifth assessment method was developed based on additional studies of intrinsic properties of sediments and drilling fluid (Ronstad and Sanford *In Press*). Previous large-scale drilling studies conducted by the Ocean Drilling Program (ODP) on seafloor sediments were deemed to be the most comparable to the deep drilling of the CBIS (House *et al.*, 2003; Smith *et al.* 2000a, 2000b). The results of ODP Legs 185 (Smith *et al.*, 2000) and 201 (House *et al.*, 2003) were used to design the techniques for microsphere and PFT assessments implemented in the CBIS.

In addition to these more standard methods, inherent characteristics of drilling fluids were also used to track intrusion of fluid into core interiors. When analyzing the dissolved organic carbon present in porewater derived from CBIS cores used in biological studies, it was found that porewaters had unique DOC signatures distinct from those of drilling muds (Sanford *et al.*, *In Press*). Profiles of DOC in porewater and drilling fluids could then be used to identify any case of potential contamination resulting from fluid intrusion into core interiors. This additional method for tracing drilling fluids provides a novel method for monitoring fluid intrusion and potential contamination of core sections during deep drilling. A second method involved characterizing acid polar components in

both drilling fluids and core porewaters, and was developed by Ronstad and Sanford (*In Press*). Similarly to DOC, profiles of acid polar components unique to drilling fluids could be used to identify the presence of fluids in any core samples. The results of this technique were made available by Ronstad and Sanford (*In Press*) as an additional cross-check of contamination assessments, and to provide information on potential contamination at depths for enumeration samples that were not covered by the aforementioned techniques.

The full coverage and results for all four techniques used to assess contamination are shown in **Table 2**. Not every core sample collected for either culturing or enumeration studies were assessed using all of the techniques mentioned. Additionally, there are some cases where one method indicated contamination in cores at a particular depth while other methods indicated no contamination. In such cases, it is again important to remember that each contamination assessment method is specific to a different indicator of contamination. For instance, microspheres may be similar in size to microorganisms, but they do not necessarily have the same surface charge. Sediments in the terrestrial subsurface can have varying charges, and could possibly be less permeable for microspheres than for microorganisms. Additionally, microspheres cannot serve as a proxy for all potential microbial contaminants because there are many known microorganisms that are smaller than 0.5 μm in diameter. Such small microbes could potentially pass through pores and micro-fractures that would exclude microspheres. In such cases, Halon 1211 can still yield a positive signal for potential contamination because it is able to pass through the micro-fractures along with drilling fluids while the microspheres cannot.

The list of potential contaminant organisms derived from clone libraries of drilling fluids was compared to sequences obtained from cultured organisms and sequences directly obtained from DNA extractions on core materials. No cultured isolates from the CBIS have yet been identified in drilling fluid clone libraries. This approach for identify potential contaminants has limitations in that clone libraries only detect the most abundant species. This means that only the most dominant organisms present in drilling fluids are

likely to be represented in clone libraries. This approach will therefore not recognize rare species present in drilling fluids that can potentially appear in cultures as contaminants. In terms of molecular studies, the approach in using clone libraries is also very conservative because it is likely that certain species of microorganisms present in the deep subsurface are from environmentally ubiquitous groups, and such groups could naturally be present in both subsurface sediments and drilling muds. This means that sequences from these groups would be eliminated from cultures, even though they may actually be indigenous members of subsurface communities.

The primary advantage of studying organisms present in the drill mud is to identify any potential contaminant organisms. No attempts to culture organisms directly from drill mud were made, although molecular studies on drilling fluids were performed with the intention of identifying potential contaminants (see Chapter 2). If an organism could be grown purely from a sample of drilling fluid, it could indicate that this same organism was a contaminant if it appeared in cultures derived from core samples. However, as drilling fluid is circulated to depth during the drilling process, it is also possible that drilling fluid could pick up organisms native to the target environment and carry them to the surface.

As of yet, no specific study on cutlureable organisms in drilling fluids has been undertaken, but such studies would be of benefit in future work. For instance, it would be particularly interesting to examine the microbial communities present in drilling fluids before and after circulating the fluid to depth. This would provide a method for testing the assumption that drilling fluids do actually pick up and carry microorganisms through the drill hole.

2.6 CONCLUSIONS

There are numerous ways in which non-native organisms can enter and contaminated samples retrieved from the deep subsurface by drilling projects (Griffin *et al.* 1997). In order to perform biological studies on cores, one must be confident that samples are free of contamination. For this reason, it is best to employ multiple methods for contamination

assessment during drilling and core retrieval. During the 2005 ICDP-USGS deep drilling of the CBIS, a suite of contamination assessments were implemented to monitor the varied ways in which contaminants can enter core interiors. These methods utilized both particulate and chemical tracers as well as studies of inherent characteristics of the various drilling fluids used. Some core samples retrieved from the CBIS were exceptionally fractured and it was impossible to avoid contamination. In all, only 6 core samples showed definite signs of contamination by at least one of the methods, and 22 were deemed free from contamination by all methods. This is one of the most extensive contamination assessment procedures implemented thus far in terrestrial deep drilling projects, and the selected suite of contamination assessment methods employed during drilling of the 2005 ICDP-USGS drilling of the CBIS allowed for the successful return of cores useful for microbiological studies. When cores free of contamination were successfully retrieved from the CBIS, enumeration studies were immediately begun. Cell counts throughout the length of the CBIS core provided a general understanding of the biological load present in materials from the surface to the base of the crater structure. Such counts would have been impossible if samples free of non-native microorganisms were not obtained.

Chapter 3: Enumeration of Microbes in Samples from Cores of the CBIS

3.1 ABSTRACT

The total number of microbial cells present in core samples retrieved from the Chesapeake Bay Impact Structure (CBIS) was determined by direct cell counts throughout the entire length of the core. Cells were visualized using DNA-binding fluorescent dyes. Optimal techniques for visualizing cells were examined in order to determine the most consistent and reliable method for enumerating cells in CBIS sediments. A selection of staining techniques and dyes were tested for estimating total cell numbers. Dyes studied included DAPI (4', 6-diamidino-2-phenylindole), Syto-9, Sybr Green I and Acridine Orange. Each of these dyes causes some amount of background fluorescence of sediment particles, and selecting a dye where cells can be accurately distinguished from this background is essential in obtaining an accurate estimate of cell numbers within a sample.

Estimating cell numbers at each sample depth provided a map of how total microbial populations change with depth, and how these numbers relate to changes in lithology, mineralogy, hydrology, chemistry and other factors associated with the impact-altered environment.

3.2 INTRODUCTION

The first step in defining the microbial biosphere in the deep subsurface of the CBIS was to estimate the total numbers of microbial cells at depth in the core. After suitable core samples were obtained using the contamination assessments described (**Chapter 2**), direct cell counts were use to determine the total number of microbial cells present in core samples retrieved from the Chesapeake Bay Impact Structure (CBIS) throughout the entire length of the core. In order to visualize cells, subsamples from cores were stained using fluorescent nucleic DNA-binding dyes. Once dyes were bound to DNA, epifluorescence microscopy was used to view and count cells. This is a sometimes difficult and time-consuming method for acquiring estimates for cell numbers, but the fidelity of staining

techniques has been proven in numerous studies conducted in a wide range of environments (Bell and Dukata, 1972; Klauth *et al.*, 2004; Francisco *et al.*, 1973; Hobbie *et al.*, 1977; Lauer *et al.*, 1981; Lebaron *et al.*, 1999; Porter and Feig, 1980).

Estimating cell numbers at each sample depth provides a map of how total microbial populations change with depth, and how these numbers relate to changes in factors such as lithology, mineralogy and chemistry associated with the impact-altered environment. Constructing the graph of total cell numbers throughout the core was an essential step in understanding the deep subsurface microbiology associated with the CBIS. It provides a baseline for determining trends in cell numbers and the general habitability of the various microenvironments present throughout the depth of the core. The enumeration profile of the core is crucial for comparison studies across fields involved with the drilling program, including microbiology, molecular biology, hydrology, geology and geochemistry.

A selection of staining techniques and dyes were tested in order to determine the most reliable and consistent approach for estimated total cell numbers. Dyes studied included DAPI (4', 6-diamidino-2-phenylindole), Syto-9, Sybr Green I and Acridine Orange. Each of these dyes causes some amount of background fluorescence of sediment particles and has unique issues associated with staining efficiency that can adversely affect enumeration estimates. Selecting a dye where cells can be accurately distinguished from this background and provide the best estimate for varied lithologies is essential in obtaining an accurate estimate of cell numbers within a sample.

3.3 BACKGROUND

In the study of microbiology, microbial communities in environmental samples have traditionally been examined by isolating organisms and cultivating them on plates (i.e. Balkwill *et al.* 1989; Benka-Coker and Olumagin 1995; Ghiorse and Balkwill 1983; Stevens 1995). With this method, plate count studies on community diversity are performed by separating taxonomic units (Dunbar 1999, Cameron 1965, 1966). Plate

counts are an inexpensive way of determining the presence of microbes in a sample and can be used to provide some estimate of biomass. However, the common perception today is that as much as 99% of microbes present in sediment samples are not cultureable using standard laboratory techniques (Kirk 2004). The total number of cultural organisms that can be obtained from a target environment is also greatly dependant on the type of growth media used (Benka-Coker and Olumagin 1995; Stevens 1995). Therefore, plate counts significantly underestimate the total number of cells present in samples (Kepner and Pratt 1994).

Plate counts cannot provide a reliable estimate of total microbial numbers, and instead provide information about the number of cultureable organisms for specific media type (Boivin-Jahns *et al.* 1996). In addition, organisms that are cultureable do not necessarily represent those that are dominant in the microbial community, either in percentage of the population or their role in geochemical processes. They are simply those best suited to growth in the laboratory under the provided conditions (media, temperature, etc.). Plate counts are most useful for enumerating specific types of viable organisms in an environment regardless of their overall importance in the microbial community (i.e. – total number of viable, iron-oxidizing bacteria in a specific environmental sample).

In response to these concerns, researchers have turned to other methods for microbial enumeration, such as direct counting of microorganisms under a microscope. Direct counting is best performed with the use of high contrast fluorescent dyes that allow cells to be easily visualized against sediment particles. Direct counting through cell staining is now often cited as the best available method for enumerating microbial cells in environmental samples (Mauclaire *et al.* 2004), and allows a more accurate understanding of total cell numbers in environmental samples than plate counts (Kepner and Pratt 1994; Yu 1995). Staining has been used to count microbes from environmental samples since the 1940s (Kepner and Pratt 1994), and numerous methods have been developed with a range of dyes for this purpose (Klauth et al., 2004; Porter and Feig, 1980; Zweifel and Hagström,

1995). Because of its ease and relatively low cost, staining is now a common practice for identifying and counting microbes in sediments and is one of the most common methods used in the literature.

In order for staining to be successfully implemented in microbial enumeration from soil and sediment samples, a number of criteria must be met. Firstly, because of the close interaction of subsurface microbes with sediments and minerals, cells are oftentimes intimately associated with particulate matter in samples. Cells can be physically attached to sediment particles through the adhesion of exopolysacharides or embedded in the microscopic pore spaces of particles, from which they are difficult to remove (Riis *et al.* 1998).

An ideal dye would stain cells exclusively and would not interact with any non-cellular materials found in samples. This is not the case for most dyes currently available. Certain dyes interact differently with varying sediment types, such as clays, sands and organoclays (Klauth *et al.*, 2004; Weinbauer *et al.* 1998). If a study site contains a range of varying lithologies, it may be necessary to adapt enumeration methods to the specific sediment type being studied. Additionally, when viewing samples under a microscope, a sediment particle can act to 'hide' a large number of cells as only the surface of the particle is visible (Clarke and Joint 1986; Gough and Stahl 2003; Kallmeyer *et al.* 2008; Schallenberg *et al.* 1989). To deal with this problem, cell counts obtained in the presence of sediment particles are often doubled under the assumption that the same number of cells should be present on the backside of particles as those in view on the topside (Gough and Stahl 2003; Kepner and Pratt 1994). Additionally, samples are often diluted to reduce the amount of sediment particles present in view under the microscope (Gough and Stahl 2003). It is often recommended that samples be diluted until ~30 cells are present in a field of view. Masking of cells by sediment has been found to be a problem in low dilutions (Schallenberg *et al.* 1989), and a large variation between replicate counts has been shown at high dilutions with fewer cells present (Kirchman *et al.* 1982). The optimal dilution

depends on the sample type, and this technique must be adapted according to target material for successful implementation.

With dilution, however, additional calculations must be made to infer total cells per unit of environmental sample (i.e. per mL or per gram of sediment). A higher dilution can result in greater weight being given to incorrectly counted cells (i.e. sediment particles falsely identified as cells or any contaminant cell accidentally introduced during the staining procedure) when calculating up to per unit numbers.

The issues caused by the close association of microbes and sediments is particularly important in enumerating samples from the deep subsurface where cell numbers can be extremely low. With such low cell densities, inaccuracies in counts caused by an inability to differentiate cells and sediments can have large effects on estimates when numbers are extrapolated up to represent larger volumes of sediment.

3.3.1 Extraction Techniques

The issues of cell/sediment interaction can cause an underestimation of cell numbers. To address this, many techniques developed for staining microbes in the presence of sediments employ a step to separate cells from sediment particles (see: Riis *et al.* 1998). This can include techniques such as agitation, sonication, enzymatic treatment or washing samples with detergents (Kallmeyer *et al.* 2008; Klauth et al. 2004).

In a recent study by Kallmeyer *et al* (2008), a new cell extraction procedure was introduced for deep subsurface sediments. Using samples from the Arctic Ocean on the IOCP Expedition 302, the researchers employed several steps to separate cells from the mineral matrix and stained samples using Sybr Green I according to the procedure outlined by Noble and Fuhrman (1998). Because this study was published shortly before completion of the CBIS work, the extraction procedure could not be implemented on the majority of samples. However, a small selection of CBIS subsamples were treated according to the procedure outlined by Kallymeyer *et al.* (2008) to see if noticeable

differences in enumeration estimates could be produced with the new extraction procedure (See Additional Separation Procedure)

None of these techniques are 100% efficient at removing cells from sediment, and the efficiency is highly dependant upon sediment types. Separation of microbes from clays and silt, for instance, has been shown to be extremely difficult and in some cases not possible (Riis *et al.* 1998). There is also an inherent loss of cells when sediment particles are removed from the sample by any method. Additionally, all of these processes can serve to damage cells, which can again cause underestimation of total cell numbers (Gough and Stahl 2003). This may be particularly important in sediments that contain a high proportion of clays (Bakken 1985), which is true for many of those collected from the CBIS. Ultimately, a balance must be found between removing sediments and the amount of manipulation that can be performed before an unacceptable loss of cells occurs (Riis *et al.* 1998).

Enumerating cells in sediments is difficult because sediment particles themselves can affect the accuracy of counts in myriad ways. Sediments that naturally fluoresce or those that are stained unspecifically by dyes can cause overestimation of cell numbers. At the same time, sediment particles can cause underestimation by blocking cells from view under the microscope. These issues must be taken in to account, and the background fluorescence of sediment present in a sample must be considered, when developing the most effective method for cell enumeration for a specific sample type.

3.3.2 Inefficiencies in Staining

Most dyes currently in use interact with a cell's genetic material (primarily DNA or RNA) in order to cause fluorescence. This means that cells must contain the material necessary for binding the dye and producing fluorescence. In bacteria, for instance, DNA is organized in a condensed region known as a nucleoid (Neidhardt et al., 1990). It should be noted that in cases where cells are moribund (inactive) or in the late stages of viral infection, they may still contain a nucleoid and therefore show fluorescence (Zweifel and

Hagström, 1995). Many staining procedures, therefore, estimate the total number of all cells containing a nucleoid and not specifically living, active cells. There are also instances in which cells lacking a nucleoid might be present, such as cell residues left behind by virus-lysed cells (Zweifel and Hagström, 1995). These cellular particles typically do not show fluorescence, but if residual DNA is present they can potentially be mistaken as intact cells. Zweifel and Hagström (1995) showed that total counts of bacterial cells in marine samples contained a large percentage of bacteria that contained no nucleoid (and were therefore not viable cells in the microbial community). It is reasonable to assume that similar 'ghost' cells as described by Zweifel and Hagström (1995) could also present difficulties in other environmental samples, such as sediments from the deep subsurface.

3.3.3 Separation of Cells from Liquids

When sediment samples are collected from the environment, they are typically added to a liquid solution for dilution and fixation/storage (see Collection of Samples below). Direct cells counts can be performed directly on suspended samples under the microscope, but more often sediments are removed from the liquid solution prior to viewing. Separation is typically performed by running suspended sediments through a filter by gravity or vacuum filtration. By filtering larger amounts of sample, the number of cells per field of view under the microscope can also be increased, providing easier viewing and reducing the effects of accidentally counting sediment particles or small amounts of contaminant cells introduced during the staining process.

In order for filtering techniques to be successful, cells must be retained on the surface of the filter and the background fluorescence of the filter and particulate matter must be low enough for cells to visualized (Hobbie et al., 1977). Additionally, it is important to achieve a homogenous distribution of cells on the filter membrane to avoid under or overestimation of cell numbers due to uneven distribution of cells across the selected fields of view.

There are a number of commercially available filter membrane types that have previously been used for direct counting of cells from environmental samples. The use of nucleopore filters to separate microbial cells from liquid samples was described as early as the 1970s (Hobbie et al., 1977). Nucleopore filters are thought to be more suitable for filtering bacteria than cellulose filters, because cellulose filters can trap a high number of cells within the filter itself making them impossible to count (Hobbie et al., 1977). Studies have shown that anodisc aluminium oxide membrane filters reduce background fluorescence with dyes such as DAPI and Sybr Green I (Jones et al. 1989; Weinbauer et al. 1998). The aluminium oxide filters also have a higher flow rate, meaning lower vacuum pressures can be used during filtering, reducing the risk of damaging cells (Kepner and Pratt 1994). However, nucleopore filters remain the most common type of filter used in direct counting studies today. This may be due to the lesser cost of Nucleopore filters (Kepner and Pratt 1994). Additionally, Nucleopore filters that are pre-stained black in colour are commercially available, providing the most appropriate background on which to visualize cells. Use of any other filters would require an additional step to dye the actual filter black before filtering samples.

Filtering samples introduces another way in which cell numbers may be reduced, and therefore yield underestimates of total cell numbers in samples. If the vacuum pressure applied for filtering is too great, cells can be damaged or destroyed. The typical pore size of filters used is 0.2 μm, and is sufficient in preventing most types of cells from passing through the filter. However, there are known microbial cells that are smaller than 0.2 μm. Small coccobacilli less than 0.3 μm in diameter have previously been identified in soil communities using electron microscopy. In a study by Bae et al. (1972), these microbes composed 72% of the soil microbial population and many of them were less than 0.08 μm in diameter. Such microbes would be too small to be seen by standard epifluorescence microscopy and would also be small enough to pass through commercially available filters.

3.3.4 Human Error

A note must also be made concerning the affects of human error in counting. Discrepancies in human vision may cause certain stains to be more suitable for viewing under the microscope simply because a researcher can more comfortably view the wavelength of light emitted by cells stained with a specific dye. This can be problematic when counts are being performed by more than one individual. Counting should be performed under a dark hood or in a dark room in order to prevent ambient light from interfering with the observer's perception of cell fluorescence. Multiple individuals should count a number of identical slides so that the estimates they produce can be compared, and any large variances in how they view and interpret fluorescing cells can be identified. These simple measures can help ensure consistency between counts.

3.3.5 Stains

Various stains are now commercially available and can be selected based on their ability to bind specific cellular components and the background fluorescence they cause in specific sediment types (Li *et al.* 2004). Because the sediment types being collected from CBIS cores were unknown, a selection of dyes were tested in order to determine which combination of dyes and techniques would be best suited for enumerating cell numbers throughout the CBIS core.

The dyes selected for testing are all nucleic acid stains, and associate with DNA or RNA molecules. None of the chemicals tested require specific reporting requirements according to regulations in North America or Europe. However, because they associate with genetic material, it can be assumed that each of the dyes are potential mutagens and should be handled with care. Important safety notes identified in the Material Safety Data Sheet (MSDS) for each dye can be found in **Appendix I.**

3.4 MATERIALS

3.4.1 Acridine Orange

Acridine Orange (AO) is a dye that has long been used for rapidly visualizing microbes in environmental samples including waters (Bell and Dukata 1972, Francisco et al. 1973, Ramsay 1978) and soils (Strugger 1948). Through the 1970s the general consensus in the microbiological community was that acridine-based dyes, in particular acridine orange (AO), were the most appropriate for use in enumerating cells in environmental samples (i.e. Kepner and Pratt 1994; Hobbie *et al.* 1977; Jones and Simon 1975).

AO is selective for nucleic acids and functions by binding both DNA and RNA (Kepner and Pratt 1994). The dye causes orange-red fluorescence when bound to RNA or single strands of DNA (excitation maximum of 502 nm and emission maximum of 525 nm), and green fluorescence when bound to double-stranded DNA (460 nm and 650 nm).

AO has been used in numerous studies of microbes in soils and sediments and is therefore a likely candidate for use in sediment samples from the deep subsurface (i.e. Bettarel *et al.* 2000, Chapelle *et al.* 1987, Frischer *et al.* 2000 and Puschell *et al.* 2007). AO has been shown to provide similar estimates for cell numbers as methods that involve the analysis of cellular components, such as membrane phospholipids or adenosine triphosphate (ATP) (Balkwill *et al.* 1988). However, studies in more recent years have shown that the dye can cause bacterium-sized particles, such as clays and colloids, to fluoresce under similar wavelengths as microbial cells (Yu 1995). It can also be exceptionally difficult to distinguish stained cells from particles in certain kinds of sediment; such as clays and detritus (particulate organic material) (Porter and Feig, 1980). Because of this, significant background fluorescence can be present when using AO, potentially causing over-estimation of cell numbers. In recent years, the use of AO has declined in favour of more modern dyes that are thought to yield better fluorescence for microbes in sediments (Kepner and Pratt 1994) (**Figure 8**).

AO is still prevalent in literature concerning the study of microbes in environmental samples, but in modern studies dealing with soil and sediment samples it is typically used as a baseline comparison alongside other dyes (i.e. Boivin-Jahns *et al.* 1996; Boulos *et al.* 1999; Mauclaire *et al.* 1999). However, although the use of AO has reduced in favour of more modern dyes (i.e. DAPI, SYBR and SYTO-type dyes), there are studies that argue against the perception that these new dyes provide better staining and signal to noise ratios. For instance, in a study by Suzuki *et al.* (1993) DAPI showed an underestimation of actual cell numbers in seawaters when compared to AO. This, of course, is dependent upon the specific researcher's interpretation of fluorescence during viewing. Because there is a large body of published literature using AO, either singly or in conjunction with other dyes, its use in staining sediments can provide a useful baseline from which to compare other methods.

3.4.2 DAPI

The problems associated with background fluorescence of AO in environmental samples have led to the more frequent use of other dyes in soil microbe studies. One of the most popular dyes has been 4', 6-diamidino-2-phenylindole (DAPI) (i.e. Glavin 2004; Weinbauer *et al.* 1998). DAPI was used for studying microbial numbers as early as 1980, and has since gained wide acceptance in the field of microbiology (Coleman 1980). In an extensive review of published enumeration studies by Kepner and Pratt (1994), it was reported that AO was used in 90% of all studies prior 1980. After 1980, 50% of studies used DAPI alone and 70% of studies since 1988 have used DAPI either alone or alongside other dyes. Today, DAPI remains commonly used in samples from a wide range of environments (i.e. Boivin-Jahns *et al.* 1996; Glavin 2004; Kuwae and Hosokawa 1999; Mauclaire *et al.* 2004; Proctor and Souza 2001; Ravenschlag *et al.* 2001, Santmire and Leff 2007, Schmid *et al.* 2007; Solera *et al.* 2001; Weinbauer *et al.* 1998; Zhang *et al.* 2007).

DAPI is an unspecific DNA binding dye, meaning that it will unpreferentially bind with DNA in living, dead and moribund (inactive) cells. DAPI shows blue-green

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fluorescence when bound to DNA (Kepner and Pratt 1994). DAPI can show background fluorescence in a range of yellow hues when unbound or bound to materials other than DNA. DAPI is often cited as showing higher image contrast for cells fluorescing against background sediment and is thought to be more specific for microorganisms than AO (Mauclaire *et al.* 2004).

DAPI has been shown to be a reliable dye for use in many types of environmental samples and is one of the most prevalent dyes cited in current literature. However, some issues with its use have been raised (Kepner and Pratt 1994; Mauclaire *et al.* 2004). Glavin et al. (2004) reported that DAPI may be unable to stain cells that do not contain a visible nucleoid region on their cell membrane, and that the dye may be less specific for DNA than previously thought. Studies have also reported that DAPI can stain particles in soil as well as DNA, thereby affecting the accuracy of cell counts (Glavin et al., 2004). In a study by Weinbauer *et al.* (1998) DAPI was shown to be inefficient for direct counts in a significant number of soil types (45% of those studied) due to high background fluorescence and unspecific staining. There is also some concern that DAPI is unable to stain some cells that do not have sufficiently high DNA content (Suzuki *et al.* 1993; Moyer and Morita 1989).

Some studies have attempted to deal with these problems by cross-staining samples with multiple dyes, such as AO or Sybr Green I (Griebler *et al.* 2001; Kuwae and Hosokawa 1999; Mauclaire *et al.* 2004). This allows the viewer to switch between multiple filters and compare the fluorescence of cells across the dyes being used. The idea is that cells are more likely to partially stain with each dye than sediments, allowing fluorescence of cells at multiple wavelengths. It should be noted that some studies have indicated that cross-staining yields significantly lower cell number estimates than staining with DAPI alone. This could be a result of overestimation of numbers when staining with DAPI alone due to counting DAPI-stained sediment particles; or it could be a case where cells are not efficiently stained by both dyes at the same time and therefore do not fluoresce at both the

excitation wavelengths being used. In either case, questions over the reliability of counts can be raised.

Despite misgivings about DAPI, it is still commonly used in current studies on soils and sediments. DAPI is also the most prevalent dye cited in microbial studies of previous drilling projects, both in terrestrial drilling and as part of the Ocean Drilling Program (i.e. Mauclaire *et al.* 2004; Parkes *et al.* 1994). As such, protocols had been developed for sediment types expected to be similar to those from the CBIS, and a set of data concerning DAPI cell counts in the subsurface is potentially available for cross comparison. For these reasons, DAPI was included as a potential dye for use on CBIS cores.

3.4.3 STYO 9

SYTO-type dyes (Molecular Probes, Inc.) are a relatively new class of dyes, and have been cited in previous studies enumerating microbial cells in different sample types (i.e. Boenigk 2004, Boulos *et al.* 1999, Gruden *et al.* 2003, Haglund *et al.* 2002, Haglund *et al.* 2006, Hymel and Plante 1998, Quéric *et al.* 2004, Sunamura *et al.* 2003). These stains have been developed in light of problems associated with DAPI staining (Klauth *et al.* 2004), and include a large number of dyes such as SYTO BC, SYTO 9 and SYTO 13 (Lebaron *et al.* 1998a). Many of these dyes are available in commercial, ready-made kits. The relative novelty of these dyes means that they have not been as thoroughly tested in terms of their application to soil and sediment samples. The use of Syto dyes for enumerating microbial cells has primarily been described for aquatic systems (i.e. del Giorgio *et al.* 1996; Lebaron *et al.* 1998a; Klauth *et al.* 2004).

SYTO 9 is used alongside propidium iodine in the LIVE/DEAD® Bacterial Viability Kit available from Invitrogen™ (Lebaron *et al.* 1998a). The LIVE/DEAD® kit distinguishes between living and dead bacteria based on the state of cell membranes. To do this, the kit uses SYTO 9, which is able to stain all cells in a sample, alongside propidium iodine, which stains only cells with damaged membranes (Leuko *et al.* 2002). Together, the two dyes allow for rapid visual estimation of both viable and nonviable cells.

Syto 9 stains cells green with an absorption of 485 nm for DNA (486 nm for RNA) and emission at 498 nm for DNA (501 nm for RNA). Syto 9 has been shown to be permeable to nearly all types of cell membranes including bacterial and mammalian cells with a low intrinsic fluorescence when not bound to DNA (Molecular Probes 2003). The suggested staining time for Syto 9 is 15 min and varying staining times ranging from 5 to 20 minutes have been shown not to effect counts (Boulos et al. 1999). There is some indication that storage of samples after staining may affect counts (Boulos et al. 1999). All stained subsamples from CBIS cores were counted immediately after staining.

In a study by Lebaron et al. (1998a), Syto 9 was shown to have high fluorescence in low salinity waters when compared to Sybr Green I and a selection of other blue nucleic acid dyes. However, in marine samples, Syto 9 fluorescence was reduced. Fluorescent noise attributed to naturally occurring fluorescent particles in seawater interfered with enumeration estimates in these high-saline waters (Lebaron et al. 1998a).

Because counts in the Lebaron et al. study (1998a) were performed by use of a flow cytometer, there is no indication of how this drop in fluorescence may affect enumeration estimates obtained by microscope counts. Individuals have the benefit of being able to identify cells based on size and morphology whereas a flow cytometer bases estimates on fluorescence intensity alone. However, lower fluorescence may cause more difficulty in discerning true cells from the background, and the results of the Lebaron et al. study should be taken into account in depths where salinity approaches levels similar to seawater.

3.4.4 SYBR Green I

The common use of AO and DAPI in studies of sediments, including the deep sea sediment studies that were used as a case study for much of the CBIS work, has lead to their continued use in current research (Fry 1990; Weinbauer et al. 1998, Parkes et al. 2000). However, newer dyes, in particular green fluorescent dyes like SYBR Green I, are now beginning to replace AO and DAPI because these dyes have been shown to have greater specificity for cells and higher fluorescence (i.e. Bedard et al. 2007, Breitbart et al.

2006, Chan *et al.* 2005, Danovaro and Serresi 2000, Danovaro *et al.* 2001, Foti *et al.* 2007, Giglio *et al.* 2003, Helton *et al.* 2006, Hewson *et al.* 2007, Kallmeyer *et al.* 2008, Luna *et al.* 2002, Lunau *et al.* 2005, Manini and Danovaro 2006, Patel *et al.* 2007, Schippers and Neretin 2006, Seymour *et al.* 2007, Zipper *et al.* 2003). In their study of staining efficiency across a range of soil types, Weinbauer *et al.* (1998) found Sybr Green dyes yielded cell counts at least an order of magnitude higher than with DAPI. However, similarly to other dyes, Sybr Green dyes have also been cited as staining soil and sediment particles as well as microbial cells (Klauth *et al.* 2004).
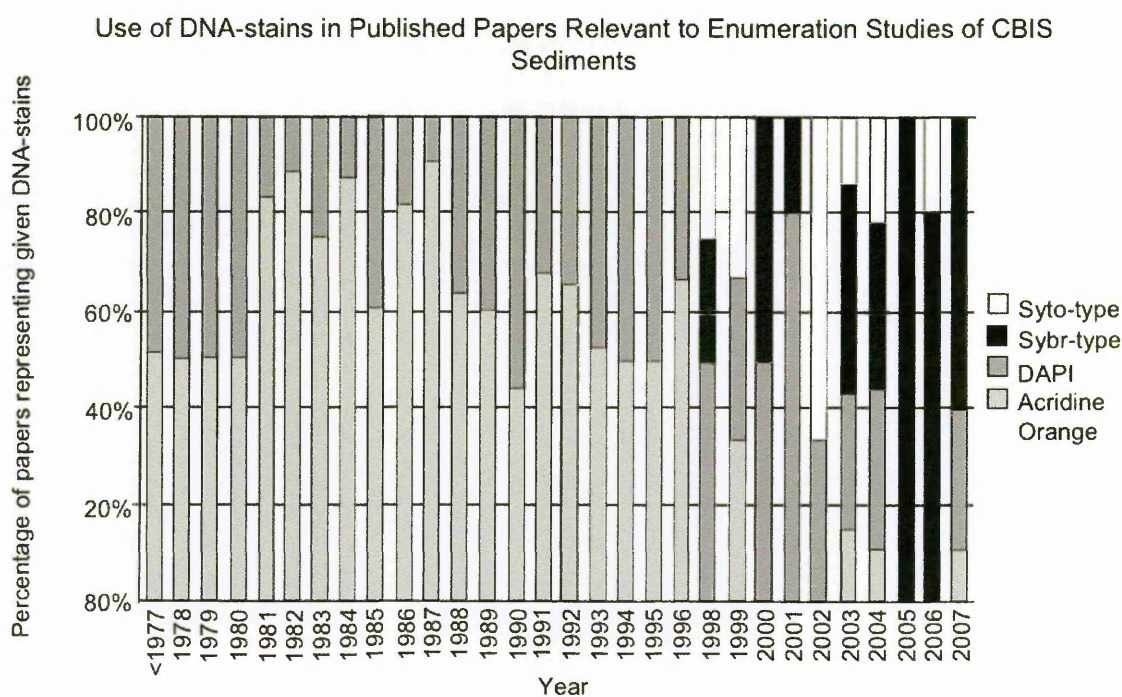
Figure 8 - Percentage of publications relevant to enumeration studies in CBIS sediments according to the types of DNA dyes used over the past 30 years. This figure is not comprehensive of all studies published using DNA-binding dyes, and represents only published studies relevant to work on direct cell enumeration in CBIS sediments.

3.4.5 Summary

No dye is perfect for the task of enumerating cells in core samples from the CBIS. There is a wealth of stains available, and for each stain there are numerous techniques that have been employed previously in the literature. Oftentimes, published studies contradict one another on which types of dye are more suitable for specific sediments. For instance, some studies claim DAPI yields higher estimates for cell numbers than AO (i.e. Kepner and Pratt 1994; Sunamura *et al.* 2003), while other studies make exactly the opposite claims (e.g.

Suzuki *et al.* 1993). Variance in the enumeration estimates cited by researchers could be due to a large number of factors. Overestimation can feasibly occur because of incorrect counting of stained or naturally fluorescing particles in samples, inefficient staining of cells due to the nature of the cells themselves, interference with staining due to the presence of environmental factors (i.e. presence of minerals, salinity, pH, etc.), contamination through improper cleaning of equipment used to prepare and stain samples and many other factors. Underestimation can conceivably be due to sediments hiding cells from view, loss of cells when removing sediment particles from a sample, destruction of cells through preparation procedures (i.e. sonication, chemical treatments, etc.), poor eyesight of individuals viewing samples, improper fixing of samples or damage to cells during fixing and storage, and many more factors.

To determine the most appropriate stains and procedures for use with sediments from the CBIS, we implemented as many methods as possible. Of course, only a reasonable amount of time could be spent testing various dyes and procedures, as it was necessary to complete enumeration studies before fixed samples degraded.

3.5 METHODS

3.5.1 Sample Collection

Core subsamples for enumeration studies were collected immediately upon retrieval of CBIS cores at the Eyreville Farm research site, Northampton County, Virginia. For a description of the drill site, see **Chapter 1**. Core sections of roughly 0.1 m were selected prior to washing of the core for geological studies (see **Chapter 2**).

On average, samples for enumeration were collected every 12 m, with the shortest distance between samples being 1.74 m and the greatest being 31.09 m (**Table 5**). The ability to successfully collect samples depended upon the quality and consistency of the material returned during coring. If core samples were highly fractured, or complete segments were not retrieved, samples were not collected due to the high likelihood of contamination (see **Chapter 2** for details). In addition, some core materials were too solid

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to crack open for subsampling. Samples were not collected from within the granite megablock section of the core from 1094.14 m to 1419.06 m. The material present in the granite megablock was solid and nearly impossible to crack open with the methods available onsite for collecting uncontaminated samples useful for microbiological and molecular biological studies (see **Chapter 2** for more details).

Table 5 Sample depths for enumeration of total cell numbers throughout the CBIS core.

Estimated Sample Depth Meters	Sample Number	Distance Between Samples
126.89	S1	8.56
135.45	S2	17.62
153.07	S3	27.40
180.47	S4	22.13
202.60	S5	22.49
225.09	S6	19.08
244.18	S7	1.74
245.91	S8	4.57
250.48	S9	25.66
276.15	S10	24.23
300.38	S11	31.09
331.47	S12	12.04
343.51	S13	8.44
351.95	S14	10.45
362.41	S15	11.89
374.29	S16	24.41
398.71	S17	12.28
410.99	S18	20.30
431.29	S19	10.36
441.66	S20	5.33
446.99	S21	2.22
449.21-449.37	S22	9.96
459.33	S23	12.31
471.65	S24	9.42
481.07	S25	12.10
493.17	S26	7.62
500.79	S27	5.18
505.97	S28,29	7.92
513.89	S30,31,32	10.24
524.13	S33	12.68
536.81	S34	5.31
542.12	S35-40	4.54
546.66	S41, 42, 43	13.14
559.80	S44	10.92
570.72	S45	12.21
582.93	S46	11.73
594.66	S47	10.33
605.00	S48	11.19
616.18	S49	10.82

627.00	S50	13.29
640.29	S51	11.98
652.27	S52	13.56
665.84	S53	15.02
680.86	S54	4.94
685.80	S55	14.81
700.61	S56	2.00
702.47 - 702.59	S57	26.00
728.53	S58	9.45
737.98	S59	11.52
749.50	S60	15.24
764.74	S61	8.90
773.64	S62	10.79
784.43	S63	12.01
796.44	S64	11.58
808.02	S65	13.08
821.10	S66	28.16
849.26	S67	1.28
850.54	S70	6.98
857.52	S71	4.39
861.91	S68	13.43
875.34	S72	12.85
888.19	S73	13.56
901.75	S69	20.00
921.75	S74	18.88
940.63	S75	1.25
958.08	S76	10.57
968.65	S77	12.20
980.85	S78	12.83
993.68	S79	11.89
1005.57	S80	9.26
1014.83	S81	10.97
1025.80	S82	14.21
1040.01	S83	11.37
1051.38	S84	12.31
1063.69	S85	8.84
1072.53	S86	11.34
1083.87	S87	10.27
1094.14	S88	
	Granite Megablock	
1419.06		
1447.28	S89	15.67
1462.95	S90	10.31

1473.26	S91	6.30
1479.56	S92	15.30
1494.86	S93	11.25
1506.11	S94	3.27
1509.38	S95	24.42
1533.80	S96	3.53
1537.33 - 1537.42	S97	24.93
1562.35 - 1562.44	S98	4.58
1567.02 - 1567.13	S99	1.47
1568.6 - 1568.47		18.84
1587.31	S100	17.71
1605.02	S101	3.71
1608.73 -		6.08

1608.78		
1614.86	S102	13.73
1628.59	S103	13.89
1642.48 - 1642.54		1.12
1643.66	S104	15.56
1659.22	S105	12.00
1671.22	S106	6.12
1677.34 - 1677.48		10.87
1688.35	S107	21.67
1710.02	S108	
Average Distance Between adjacent samples:		12.38

Core sections were handled using nitrile or latex gloves and immediately transferred to a UV sterilized workspace. The core section was then transferred to a sterile piece of aluminium foil. Samples of drilling mud on the outside were collected for contamination analysis (see **Chapter 2**). The outside of the core was then scraped away using a UV and flame sterilized knife and spatula. The core was then transferred to a fresh piece of sterile foil and gloves were discarded and replaced with a fresh pair of UV sterilized gloves. If the core material was such that the exterior could not be paired away using a knife and spatula, drill mud was carefully wiped away from the exterior surface. The core was then passed quickly through a flame and briefly wiped with ethanol.

Once the exterior of the core was safely removed, the core was cracked open using a UV and flame sterilized hammer and chisel to expose interior sections. Samples were collected from as close to the centre of the core as possible. Two subsamples were collected from each core and immediately added to 20 mL of a solution composed of artificial seawater (ASW: NaCl 27.4 g, KCl 0.77 g, $MgCl_2$ 11.21 g, $NaHCO_3$ 0.202 g, $CaCl_2$ 2.4 g dissolved into 1 L filter sterilized deionized water) (Cragg *et al.* 1990) and 2% formaldehyde (i.e. Mauclaire *et al.* 1999). Bottles of 20 mL ASW/formaldehyde solution (

Appendix III – Additional Solutions) were prepared in a sterile laminar flow hood at the Open University in pre-autoclaved 25 mL metal cap bottles (**Figure 9**) and shipped to the drill site for use in collecting samples. When subsamples were selected onsite and placed in the metal cap bottles, the bottles were immediately resealed and prepared for transport to the Open University in the United Kingdom where enumeration studies were performed. Epifluorescence microscopy was carried out using a Leica DMRP microscope, a minimum of 200 fields of view were counted for each depth. The numbers reported here are the mean values per depth.

Figure 9 - CBIS core sediments samples collected and stored in sterile metal-cap glass bottles.

3.5.2 Acridine Orange

Initial attempts at staining samples were performed with Acridine Orange. Although use of this dye has declined in recent years due to concerns over its accuracy as compared to more modern dyes, AO is still common in the literature and provided an appropriate starting point from which to compare additional dyes. The initial procedure used for AO staining was adapted from Cragg *et al.* (1990) and was successfully used with samples from deep seafloor sediments during the ODP Legs 112 and 139 (Cragg *et al.* 1990; Cragg and Parkes 1994).

Fixed core subsamples were gently agitated by inversion for one minute, or until sediments were re-suspended in solution. 100 μL of suspended sediment was removed and placed in a pre-autoclaved Eppendorf tube. The sediment was then vortexed briefly and

added to 10 mL of filter-sterilized ASW. AO was added to a final concentration of 5 mg/L. Samples were placed in a dark box and allowed to stain for 10 to 30 min. The sample was then filtered through a 25mm, 0.2 µL Nucleopore filter by gentle suction. The filter was then rinsed with 12.5 mL of a citric acid buffer composed of two solutions (A+B), [100 mL of Solution A (10.5 g Citric Acid in 500 mL filter sterilized deionized water) was added to 59 mL Solution B (2 g NaOH in 500 mL filter sterilized deionized water) and filter sterilized (Cragg *et al.* 1990). Filters were then mounted under a coverslip using a minimum of emersion oil.

Multiple dilution factors of sediments were attempted, ranging from a starting sample of 10 µL of suspended sample to 1 mL of suspended sample. This was done to test the fluorescence of AO-stained cells in the presence of varied concentrations of sediment particles.

3.5.3 DAPI

Since becoming commercially available, DAPI has become more commonly used than AO (Kepner and Pratt 1994; Weinbauer *et al.* 1998). DAPI was the second choice for staining CBIS samples due to the frequency of its use in past and current published studies. For DAPI staining, organisms were observed using an excitation filter with a bandpass of 340-380 nm (filter cube A) and an emission filter with longband cut-off >425nm. For DAPI methods used, only visible cells were counted and numbers were not altered to account for cells hidden by sediments. Because most samples were sonicated and filtered, it was assumed that the majority of cells were detached from sediment particles and the concentration of particles in the field of view was low enough that concealment of cells was not an issue.

Method 1
The first attempt at staining of CBIS sediments was performed by preparing a DAPI stock solution of 0.1 mg/mL. Sediment samples were re-suspended by gentle agitation and a 1 mL aliquot of suspended sample was placed in a pre-autoclaved Eppendorf tube. 2 µL of

DAPI stock solution was added and the sample was placed in a dark box for 30 min. The sample was then filtered through a 25mm, 0.2 μm Nucleopore filter using gentle suction. The filter was washed with 2-5 mL of filter sterilized deionized water and then mounted under a coverslip with a minimum of emersion oil.

Method 2
A second attempt at staining core samples with DAPI was adapted from a procedure obtained from the Voytek Microbiology Laboratory at the USGS. The procedure showed excellent fluorescence for many of the sediment types and was adopted as the primary method for obtaining cell enumerations from CBIS subsamples.

A stock solution of DAPI was prepared by adding 2 mg of DAPI to 20 mL filter sterilized deionized water. A staining solution (Solution 1) with a final concentration of 5 μg/mL DAPI and 0.1% Triton X was prepared in Phosphate Buffered Saline solution (PBS) (20 μL Triton X, 1 mL of DAPI stock and 1.9 mL of 10X PBS, 17.1 mL filter sterilized deionized water). A second staining solution (Solution 2) with 5 μg/mL DAPI and 0.01% Triton X was also prepared (2 μL Triton X, 1 mL of DAPI stock, 1.9 mL of 10X PBS, 17.1 mL filter sterilized deionized water). Stock DAPI (powdered) and all staining solutions were stored at 5°C when not in use.

Core subsamples stored in formaldehyde solution were gently agitated by inversion for one minute, or until sediments were re-suspended in solution. One mL of suspended sediments was then added to 1 mL of Solution 1 in a pre-autoclaved sterile glass vial. An additional 1 mL aliquot of suspended sediment was then removed and placed in a second pre-autoclaved glass vial for drying and weighing to obtain an estimated mass of sediment being counted. The sediment/Solution 1 mix was then sonicated for one minute at 3 RMS (Microson ultrasonic cell disruptor). In order to avoid heating, the sample was sonicated in five second intervals over a total of one minute. Samples were then placed in a dark box and the sediments were allowed to settle out of solution (~10 minutes). Supernatant was removed (~2mL), put into a separate pre-autoclaved sterile bottle and placed on ice in the

dark. One mL of Solution 1 was then added to the original bottle and the sample was re-sonicated for an additional minute. The sample was again allowed to settle out of solution. The supernatant (~1 mL) was removed and decanted in the previously collected supernatant and placed back on ice. A final 1 mL of Solution 1 was then added to the original sample and sonication, settling and removal of supernatant was repeated. The final collection of supernatant was decanted in the previously collected supernatant to yield a total of ~4 mL.

The ~4 mL of collected supernatant was then sonicated for 1 min (as above). Following sonication, 1 mL was immediately added to a pre-autoclaved filter column prepared with a 0.2 μm Nucleopore filter. Two mL of Solution 2 was added to the column and the column was then placed in a dark box to stain for 30 minutes. The entire solution was filtered through the column by gentle suction. The filter was then mounted on a glass slide and a drop of fluorescence free emersion oil was added on top. A cover slip was placed on top and the slide was viewed at 1000x magnification using a Leica DMRP microscope equipped with an excitation filter with a bandpass of 340-380 nm (filter cube A) and an emission filter with longband cut-off >425nm.

A total of 50 fields of view in wide angle mode (0.2 mm diameter) were counted. If cell numbers were greater than 50 cells per field of view, the field of view was reduced to a narrow angle (0.05 mm diameter). This was done to reduce inaccuracy in counting due to cells losing fluorescence under UV light. If a large number of cells were present, it was more likely that cells could lose their fluorescence before being counted. This would then cause an underestimation in the total number of cells per field of view.

3.5.4 DAPI/SYBR Green I Cross-Staining

A cross-staining technique using DAPI and Sybr Green I was also implemented for depths that showed high background fluorescence. The cross-staining of aquatic sediments using DAPI and Sybr Green II has been previously described by Griebler *et al.* (2001), and was the basis for attempting a counterstain technique using Sybr Green I.

A Sybr Green I staining solution was made by diluting Sybr Green I by 5×10^{-4} from the original stock solution (Molecular Probes). Stock and diluted Sybr Green I was stored at -20°C when not in use. Samples were prepared according to DAPI Method 2 (above). Before placing filter columns into the dark box for staining, 5 μL of the Sybr Green I staining solution was added to the columns. Following staining for 30 minutes, samples were filtered by gentle suction and filters were viewed under epifluorescent microscopy (excitation filter with a bandpass of 450-490 nm (filter cube I3) and an emission filter with longband cut-off >515nm).

3.5.5 SYTO 9

Attempts to stain samples were also performed with the more modern dye, Syto 9. Although Syto 9 has not been tested as extensively as AO and DAPI with regard to sediments, it has been shown to yield excellent results with aqueous samples. For Syto 9 staining, an excitation filter with a bandpass of 450-490 nm (filter cube I3) and an emission filter with longband cut-off >515nm was used.

Method 1

A stock solution of Syto 9 was prepared by adding 3.3 μL of Syto 9 (Invitrogen) to 100 μL filter sterilized deionized water (1.67 μM). The Syto 9 was kept at -20°C when not in use. Samples were agitated gently to resuspend sediments in the formaldehyde solution and 1 mL of suspended sample was withdrawn and placed in a pre-autoclaved sterile Eppendorf tube. A second aliquot of sample was removed for drying and weighing. Samples were stained with 2 μL of the Syto 9 stock solution for 15 minutes in a dark box. Samples were then filtered onto black Nucleopore filters (0.2 μm). Filters were then washed twice by filtering 2 mL of sterile deionized water through the filter column by gentle suction. Filters were then transferred to glass slides for cell counting under the microscope as with DAPI staining (above).

Method 2

Core subsamples were agitated gently for one minute by inversion or until all sediment had been re-suspended in the formaldehyde solution. Bottles were then left to sit for ten minutes to allow the majority of sediments to resettle on the bottom of the metal cap bottle. Samples were then re-agitated gently until the liquid solution was slightly opaque. The opacity of the solution was kept minimal to ensure that the amount of sediment particles was such that sediment would not completely fill the field of view when viewed under the microscope. Ideally, concentration of sediment was such to achieve an even distribution of sediment particles where individual particles did not overlap when viewed.

200 µL was then removed from the metal cap bottle and placed in a pre-autoclaved sterile Eppendorf tube. A second 200 µL aliquot of suspended sediment was then placed in a second Eppendorf tube to be dried and weighed later to provide an estimate of the mass of sediment being counted. 2 µL of the Syto 9 stock solution was then added to the first Eppendorf tube for staining, yielding a dye concentration of 5 mM. The sample was then placed in a dark box for 15 minutes. After 15 minutes of staining, 10 µL of the sample was placed on a glass slide and viewed under epifluorescence microscopy.

Method 3

A third method for Syto 9 staining was performed using an adaptation of DAPI Method 2 (above). This was done in an attempt to compare the staining efficiency of Syto 9 with DAPI more directly. Additionally, because such a small amount (200 µL) of sample was analyzed in Syto 9 Method 2, it was thought that analysis of a greater portion of sample would yield more representative results when estimating total cells per gram of sediment. Syto 9 Method 3 followed the same protocol as the DAPI Method 2, only DAPI was substituted with Syto 9 in the preparations of Solutions 1 and 2.

3.5.6 Additional Separation Procedure

An attempt to compare an additional cell separation procedure developed by Kallmeyer *et al.* (2008) was performed in order to compare the results of the new procedure to those obtained with the aforementioned techniques.

Kallmeyer *et al.* (2008) recommended the use of Sybr Green I based on the staining procedure outlined by Noble and Fuhrman (1998). For our study, we used both Sybr Green I and DAPI staining to compare the results of the Kallmeyer *et al.* extraction method to the aforementioned DAPI staining procedure (above).

3.5.7 Calculations of Cells per Gram Sediment

Estimates for the amount of sediment being analyzed during cell estimates were obtained by drying and weighing aliquots of suspended sediment taken during staining of each sample. Aliquots were collected at the time of staining in pre-weighed glass vials. Aliquots were dried overnight in an oven. Samples were then immediately transferred to a desiccator to prevent any condensation from forming in the glass vials. Once completely desiccated, samples were quickly weighed. The weight of the pre-weighed vials was then subtracted from the total weight of vial and sediment to obtain an estimate for the amount of suspended sediment at the time of staining. Aliquots of suspended sediment were taken for every individual count obtained with the selected staining methods to account for any variations in weight between sediment types and staining aliquots. Sediment weight estimates were used to estimate the number of cells in a gram of sediment. Weight estimates also provided a means for calculating the dilution of sediment being counted in each staining attempt. The optimal dilutions for sediments ranged from 1/100 to 3/100, although a dilution of 1/100 was found to be the most appropriate for DAPI Method 2 in the majority of sediments encountered in the CBIS.

For DAPI Method 2, the primary counting method selected, the area of the filter used for filtering samples was 200.96 mm^2. The field of view in wide angle under the microscope at 100x magnification was 0.0314 mm^2. 50 fields of view covered a total area

of $1.57mm^2$, which represents (1.57/200.96) 1/128 of the total area. The estimated number of cells on the entire area of the filter then corresponds to 128 x the total cells in 50 fields of view (value dubbed Ans 1). This value (Ans 1) represents the total number of bacteria in 1 mL of filtered supernatant from the treated sample. So, (Ans 1) x 4 is equal to the total bacteria in 4 mL of supernatant (Ans 2). The total number of cells per gram of sediment could then be found by (Ans 2)/(g desiccated sample aliquot).

3.5.8 Estimating Total Cells based on Microscopic Counts

Total numbers of cells were counted in 50 fields of view for each sample prepared by all methods. Based on the results of the methods tested, Syto 9 METHOD 2 was selected for initial counts of the upper core sections and DAPI METHOD 2 was selected for counts throughout the entire core. For each depth, a minimum of two replicate counts were made from each of the two subsamples collected onsite at the Eyreville Farm. This means that a minimum of four samples were stained and counted per depth, providing an estimate based on 200 fields of view. The numbers presented in this text are the mean of all 200 fields of view counted at each depth.

Because cell counts were calculated up from only a small portion of sample viewed under the microscope, a 'detection limit' was set to account for samples that contained too few cells to yield reliable enumeration estimates. In sediments with extremely low cell numbers, introduction of even a few contaminant cells during sample preparation could skew the total number of cells estimated per gram of soil. To account for this, a detection limit of $5x10^4$ cells per gram sediment was set. Any estimates that fell below $5x10^4$ were considered too low to be considered reliable. This number is equivalent to roughly 4 cells in 50 fields of view.

Blank samples of the formaldehyde solution were also stained to check for contaminants present in the solution before fixing core samples. Blanks were also prepared alongside sediment samples in order to check for contamination introduced during the

staining procedure. A minimum of two blanks were tested each time a new stock solution of the target stain was prepared. All counts on blank samples were negative.

3.6 RESULTS

Initial counts with all dyes were performed on a selection of sediments from the upper regions of the core between depths of 125 to 450 meters. Previous studies on terrestrial soils show a decline in cell numbers with depth, and it was assumed that sediments from this region of the core would be richer in cell numbers due to their proximity to the surface biosphere (Colwell 1989). We selected these sediments to ensure that tests of the various dyes were not performed on sediments that contained no cells.

After initial counts to determine the most appropriate methods for enumeration were completed, all subsequent counts were performed on random samples. Sample numbers and depths were not recorded until after staining and counting had been performed. Graphs of cell numbers were not constructed until the majority of all samples were counted. This prevented any anticipated trends in microbial numbers from affecting counts performed by individuals. Select depths were also counted by multiple individuals and numbers were compared in order to assess whether or not differentiation between cells and fluorescing sediment particles was sufficient to produce reliable counts over multiple replicates of stained samples.

3.6.1 Acridine Orange

AO showed a great deal of background fluorescence in the initial sediments tested. Cells were difficult, if not impossible to discriminate from small sediment particles (Data not shown). The difficulty in counting cells was similar at multiple dilutions of samples. Attempts to enumerate cells using AO showed that the dye provided less accuracy than concurrent tests with DAPI and Syto-9. Because these dyes provided better fluorescence of cells in CBIS sediments, further tests with AO were not attempted.

3.6.2 SYTO 9

The first attempts to stain CBIS samples using Syto 9 were performed using Method 1 (above). Similarly to AO, it was difficult to distinguish cells from background fluorescence on filters at a range of dilutions. Sediment particles were present on the filter in high densities and background fluorescence was such that cell counting was not possible (**Figure 10**).
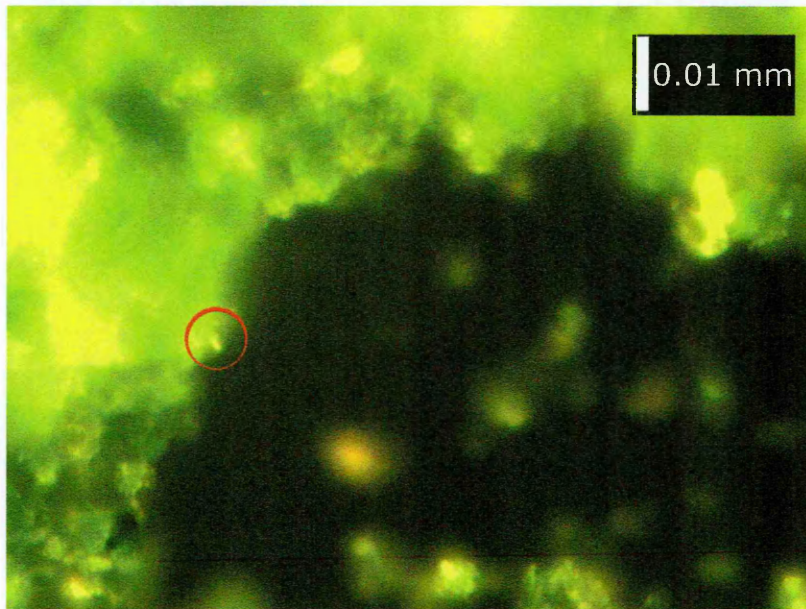
Figure 10 - CBIS sample from a depth of 300.4 meters stained using Syto 9 METHOD 1. A potential cell is circled in red.

A second attempt to stain cells was made with Syto 9 using the least amount of sample manipulation as possible prior to staining (Method 2 above) in order to determine if the poor results obtained with Method 1 were in some part due to cell disruption/ destruction during filtering. Syto 9 Method 2 involved no potentially damaging steps such as separation and filtering, and cells were therefore stained directly in their preserved state. This, however, means that no cells were forcibly detached from sediment particles and were therefore more difficult to distinguish individually. Under white light, the entire field of view was covered with sediment particles of varying size. Under fluorescence, a small fraction of sediment particles were visible. Some cells were present free-floating in the liquid, but most cells were associated with sediment particles (**Figure 11**). Dilutions for

Syto 9 Method 2 were 1/25, but only 200 μL of suspended sediment was stained (an average of 6.9 mg of sediment).
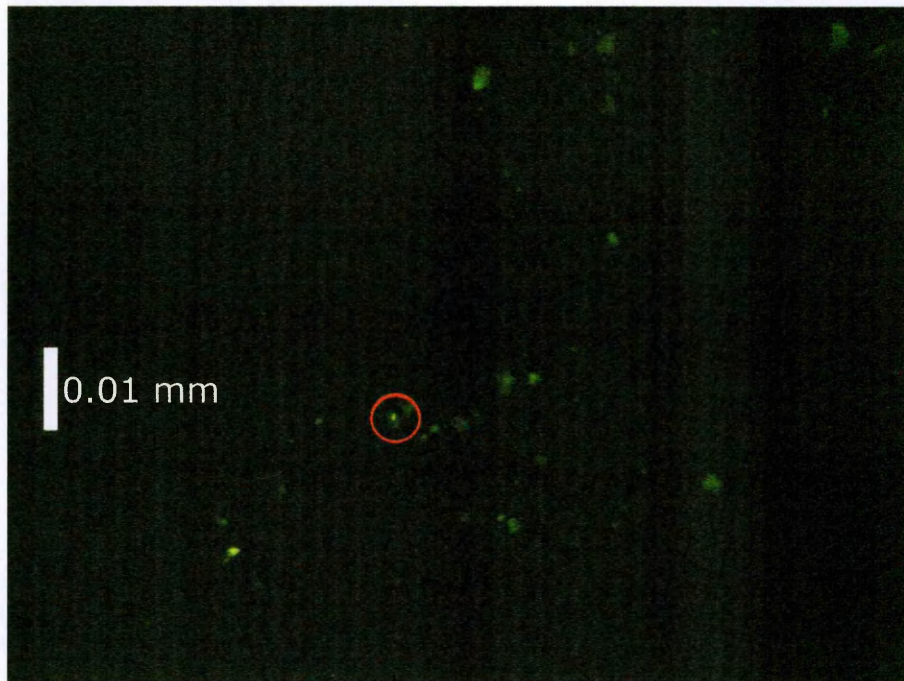
Figure 11 - Sediments and cells stained using Syto 9 METHOD 2 at a sample depth of 300.4 meters. A potential cell is circled in red.

Syto 9 Method 3 was a further attempt to use Syto 9 alongside cell separation procedures in order to reduce potential overestimates of cell numbers due to the presence of small, microbial-like sediment particles. This method was also performed to test how Syto 9 counts compared to DAPI counts when the exact same preparation procedure was performed with samples. Overall, staining using Syto 9 Method 3 was successful. However, the level of fluorescence in cells was low and cells appeared only slightly brighter than background sediment particles (**Figure 12**). Because of this, enumerations estimates using Syto 9 Method 3 were significantly lower than those obtained with DAPI Method 2 (data not shown).
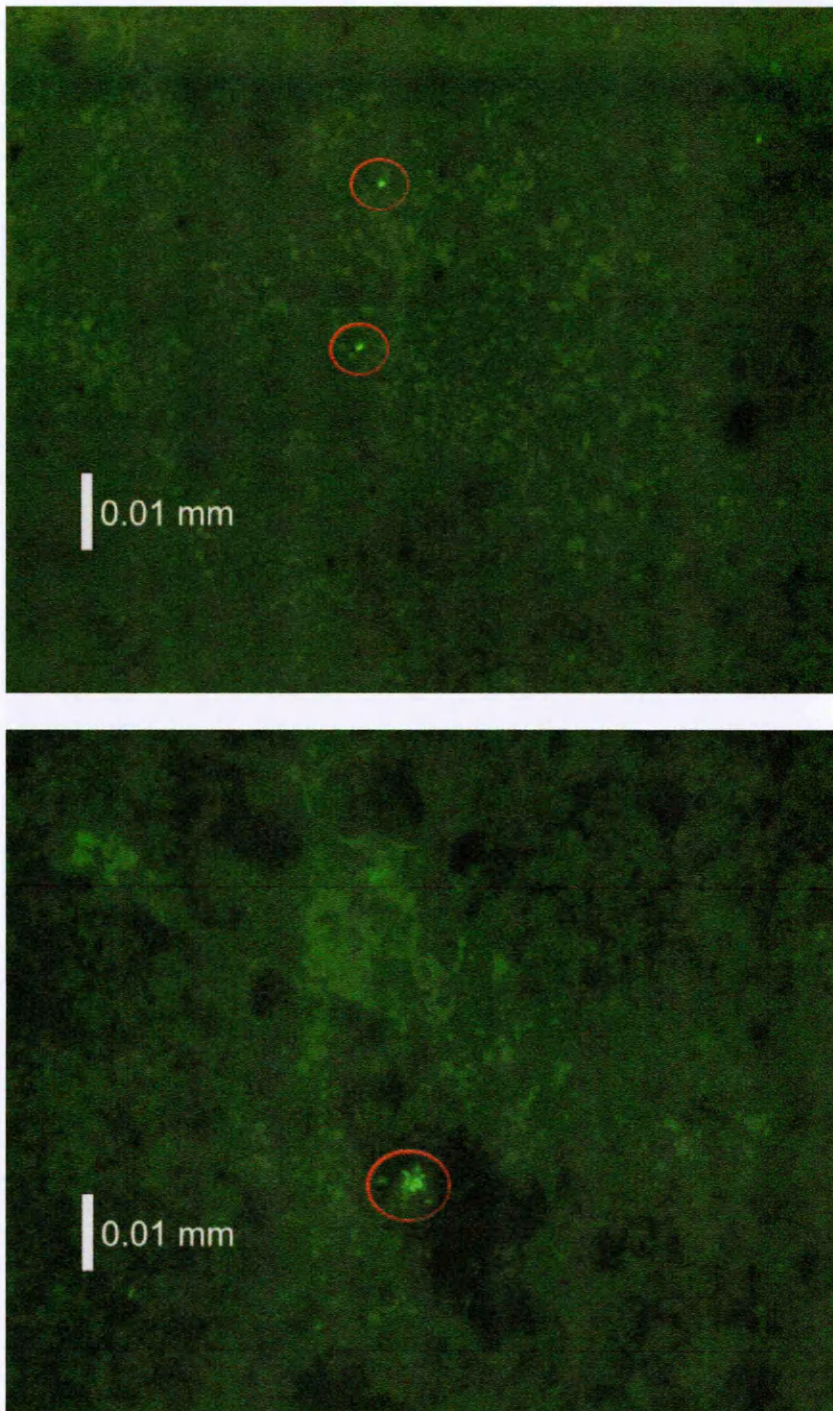
Figure 12 - Stained cells using Syto 9 Method 3 from a sample depth of 245.9 meters (two replicates). Cell fluorescence is low, and numbers are significantly lower than those from identical depths when stained with DAPI Method 2.

3.6.3 DAPI

Studies suggest that DAPI can stain samples effectively after 5 minutes, and that

increasing the staining time does not alter the ease or reliability of visualizing cells (Porter

and Feig, 1980). DAPI fluorescence appeared to be stable for up to three minutes under the

microscope, which is consistent with previous studies (Porter and Feig, 1980). Although DAPI-stained cells have been shown to remain countable after 24 weeks when stored at 4°C (Porter and Feig, 1980), slides were counted immediately upon preparation to ensure accurate counting of cells present at the time of staining.

DAPI Method 1 showed a great deal of background fluorescence and cells were extremely difficult to distinguish from sediment particles. At most dilutions, filters contained too much sediment to enable counting. At low dilutions, some cells were visible but background was too high to achieve similar counts in multiple replicates (data not shown).

DAPI Method 2, however, showed excellent fluorescence of stained cells in many of the sediment types encountered in the CBIS. Initial counts of cells showed similar results across multiple replicates of samples and with viewing by multiple individuals. Cells appeared bright blue against a background of yellow/orange sediment (**Figure 14** through **Figure 17**). Most of the sediment was present on filters after staining as a thin film. Particulate sediments were often large or irregularly shaped, and were easily distinguishable from actual microbial cells. For these reasons, DAPI Method 2 was selected as the method of choice for staining samples throughout the entire length of the CBIS core.
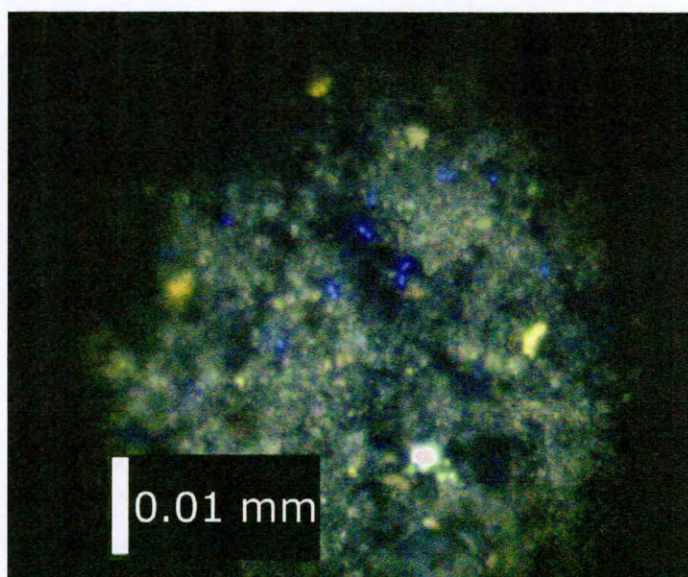
Figure 13 - CBIS sample from a depth of 127 meters stained using DAPI METHOD 2.
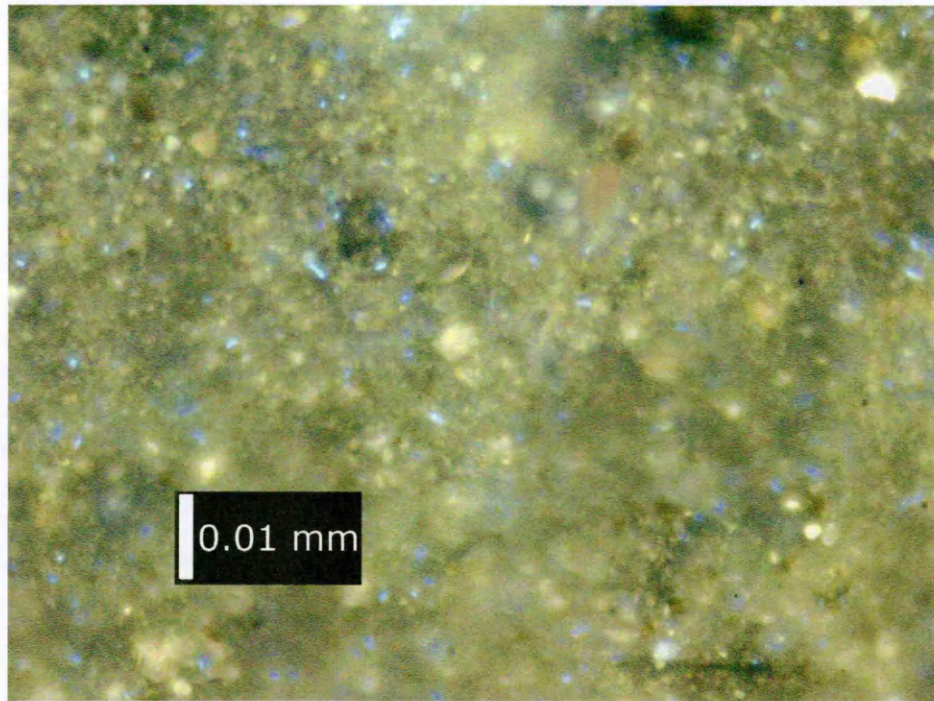
Figure 14 - CBIS sample from a depth of 300.4 meters stained using DAPI METHOD 2.
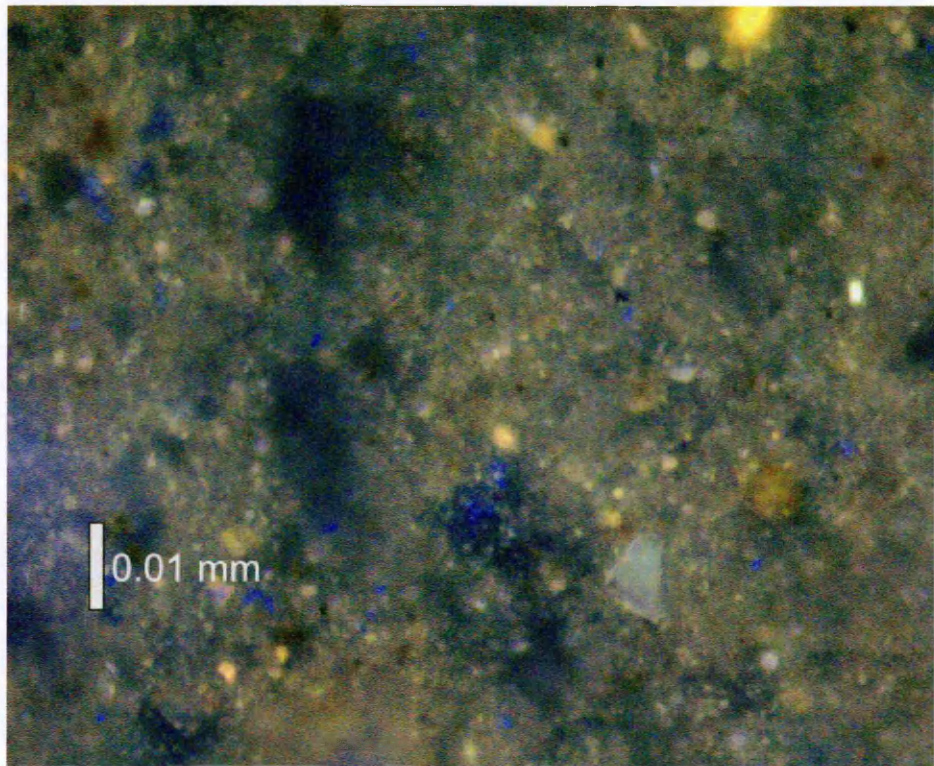
Figure 15 - CBIS sample from a depth of 331.5 meters stained with DAPI METHOD 2.
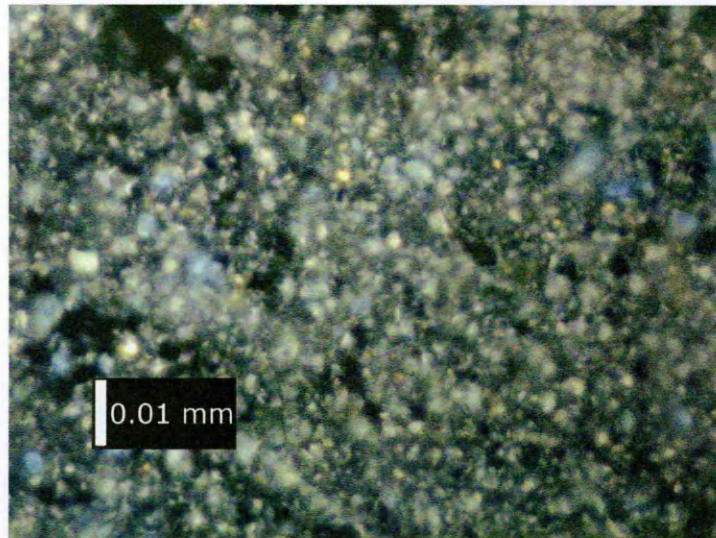
Figure 16 - CBIS sample from a depth of 875 meters stained using DAPI METHOD 2.
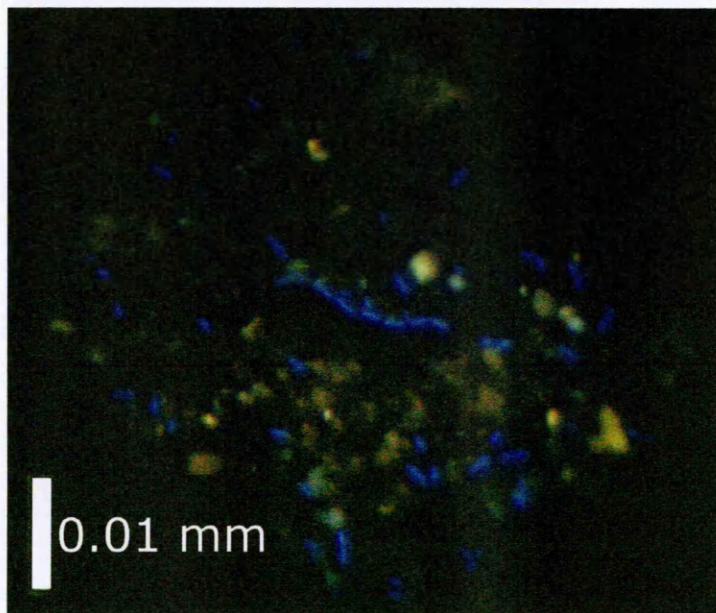
Figure 17 - CBIS sample from a depth of 1644 meters stained using DAPI METHOD 2.

3.6.4 Cross-Staining with Sybr Green I

Cross staining of samples using DAPI and Sybr Green I was performed on a range of samples from depths where DAPI Method 2 resulted in counts that were near or below the detectable limit. This was to test whether or not low cell numbers in these samples was due to staining inefficiencies of DAPI in sediments at these depths. It was also essential to be sure that cells were being counted accurately when present in such low numbers, because even a single mis-counted cell could greatly affect total cell numbers when calculating up to estimate cells per gram of sediment.

Cross staining allowed any cells identified with DAPI to be cross-checked against Sybr Green I. If a cell fluoresced with both dyes, it was deemed to be a microbial cell and not simply a fluorescing sediment particle. Sybr Green I could also then be used to identify the number of potential cells in sediments that did not stain with DAPI. Very few cell-like objects were seen fluorescing exclusively with Sybr Green I, indicating that cell numbers at these depths were indeed below the detectable limit. Additionally, counts performed with cross-staining on samples that showed cell numbers near the detectable limit yielded similar cell number estimates than DAPI alone (data not shown).

3.7 DISSCUSSION

The 2005 drilling of the CBIS was the first sampling of its kind. Because the types and condition of sediments present in the impact structure were unknown prior to drilling, it was important to employ a range of dyes and techniques in order to find a suitable way in which to enumerate cells throughout the core. The dyes selected for initial experiments were an older, commonly cited dye (AO), the most oft-cited dye in contemporary studies (DAPI) and two newer dyes that have not been as thoroughly tested for use with sediments (SYTO 9 and SYBR Green I).

The techniques ranged from the most minimal treatment of samples as possible to those that require more manipulation of samples during the staining process; including both physical and chemical treatments. Multiple dilutions were also attempted in order to determine the most appropriate dilution with which to achieve an even spread of cells with as small amount of sediment as possible to avoid masking of cells. Finally, cross-staining with two dyes was also employed in an attempt to more accurately count cells in sediments that showed a high background fluorescence or extremely low numbers of cells. It was essential to select the most appropriate stain and technique for enumerating cells throughout the core as quickly as possible after samples were collected because the effects of long-term storage of samples on enumeration estimates were also unknown.

The comparison studies performed on epifluorescent dyes suggest that no single dye is completely effective through mixed lithologies, such as those encountered in an impact crater (and other subsurface environments with mixed geological strata), and complementary methods may be needed. Understandably, changes in the composition of sediments throughout the core can cause varying amounts of background fluorescence at different depths, meaning that specific dyes may be better suited for the varying materials. In this study we found that staining with SYTO-9 Method 2, DAPI Method 2, and cross staining with DAPI and SYBR Green I provided the most robust methods for estimating total cell numbers throughout the 1766.3 km-deep CBIS core.

3.7.1 DAPI v Syto 9

Enumerations with SYTO-9 Method 2 were significantly higher than those with DAPI Method 2. There was no separation of cells and sediment in Syto 9 Method 2 and it is likely that this method provided overestimation due to the presence of fluorescing sediment particles. Syto 9 counts were performed through the upper portion of the core when cell numbers were high. DAPI Method 2 provided more differentiation in fluorescence between cells and sediment particles, and was more appropriate for cell counts in sediments where cell numbers were low. Because of this, DAPI Method 2 was selected for performing counts throughout the length of the core.

On average, counts with Syto 9 Method 2 exceeded DAPI counts by two orders of magnitude (**Figure 18**). This is a substantial difference in numbers across the two dyes, and the mean difference between counts performed with Syto 9 and DAPI in the region of the core corresponding to logarithmic decline was 128 times greater using Syto 9 Method 2. Additionally, counts using Syto 9 Method 2 remained high (> 10^7) past the transition into the resurge deposits within the crater structure (**Figure 6**) where few or no cultureable organisms were obtained (**Chapter 4**) and DAPI Method 2 counts were below the detectable limit.
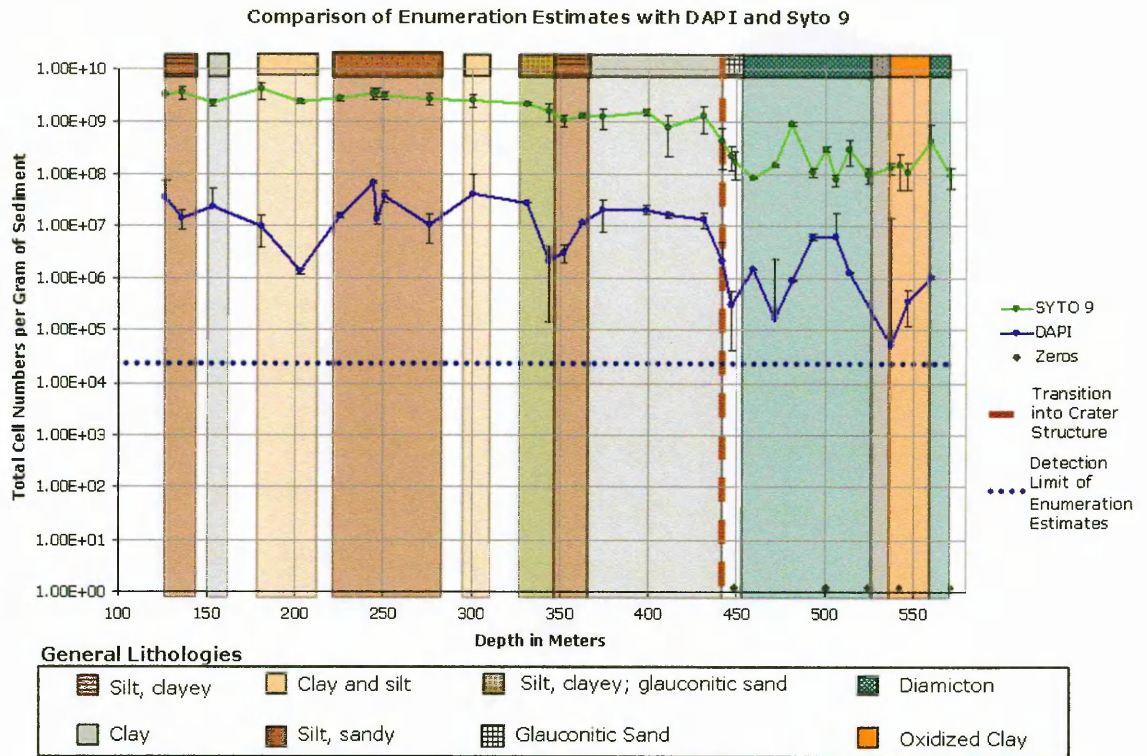
Figure 18 - DAPI and Syto 9 counts at identical depths plotted against the lithologies of sediments that were sampled. Each point on the graph represents the mean value obtained from two replicate counts each on two subsamples – totalling four separate counts per depth of sediment. Zero numbers for counts below the detection limit with DAPI are indicated by grey diamonds. Error bars show one standard deviation from the mean for all counts at the given depth.

Both methods showed a similar trend in logarithmic decline with depth. Trends within lithologic units were more pronounced with DAPI. For instance, variation between Syto 9 Method 2 counts within the silt, sandy block at depths ~220-280 is relatively low, with mean values at these depths varying from 2.7×10^9 to 3.6×10^9. DAPI counts within the same lithologic unit range from 6.1×10^6 to 7.0×10^7.

Varying numbers within similar sediments are not entirely surprising because microbiological communities in sediments can vary dramatically along with small-scale changes in sediment type. Sampling from cores does not necessarily provide a uniform representation of the sediment present in an entire core section. However, variations in orders of magnitude, such as those seen with DAPI, may be related to changes in small-scale mineralogy or geochemistry of the sediments.

As discussed, there are many reasons that staining methods and dyes can cause both overestimation and underestimation of total cells. The study by Schallenberg *et al.*

(1989a) showed that DAPI can yield an underestimation of cell numbers because of the varying affects of dye concentration and masking of cells by sediment particles. The effects of varied sediments on the staining efficiency of Syto 9 are not well documented. Additionally, the DAPI method employed in this study required a sonication step, which could feasibly damage cells and thereby reduce the number of fluorescing cells in a sample.

Syto 9 has previously shown variations in fluorescence intensity between freshwater and seawater samples, and the concentration of brines within the CBIS could potentially have some effect on staining efficiency (Lebaron *et al.* 1998a). There is also some indication that Syto 9 may show decreases in fluorescence intensity when used to stain samples fixed with formaldehyde (Lebaron 1998a). If cell fluorescence is low, it becomes more difficult to distinguish between cells and particles.

In some previous studies, DAPI has shown high variations in staining efficiency across varying sediment types (Shallenberg *et al.* 1989). However, areas where large variations occurred, or where cell numbers were low, were cross-stained with Sybr Green I in order to verify the estimates by using multiple dyes on identical samples.

3.7.2 Enumerations and Microbiology of the CBIS

Impact events are thought to have profound affects on surface dwelling organisms on Earth. The effects of impact on the subsurface biosphere, however, are not documented. Creating a profile of microbial cell numbers throughout the CBIS core was the first step in understanding the effects of impact on the biology of the terrestrial subsurface. Enumeration profiles were compared to data on lithology, porosity and chemistry associated with core sections and provided by researchers at the USGS in Reston, Virginia, and members of the project team of the 2005 ICDP-USGS deep drilling of the CBIS.

Cell enumerations identified three microbiological 'zones' through the length of the core (**Figure 19**). These zones correspond to well-defined lithological sequences that are linked to impact processes. Zone 1 (127 to 867 m) shows logarithmic decline through the post-impact sediments (zone 1a; 127-444m) into the diamicton interpreted as resurge

deposits (zone 1b; 444-618 m) and sediment megablocks with diamicton intercalations (zone 1c; 618-867m). This zone stretches from the surface through the post-impact section composed of upper Eocene to Pliocene marine sediments and Pleistocene non-marine sediments and across the transition into the crater structure, where impact tsunami resurge sediments and sediment megablocks are present.

Zone 2 of the CBIS core (867 to 1096 m) is defined as a quartz sand-rich zone. The bottom of this zone is composed of boulders and blocks that may have been sterilized by the thermal pulse generated by the impact. Enumeration estimates in this zone of the core were below the defined detection limit for DAPI Method 2. Enumerations were also below the detection limit when samples were cross-stained with DAPI and Sybr Green I.

Zone 3 (1397 to 1766 m), which sits below the granite megablock where enumeration samples were not collected, showed increasing microbial numbers. This zone corresponds to suevite (an impact breccia with shocked and unshocked clasts and melt particles) and underlying schist (a metamorphic rock) and pegmatite (igneous rock with coarse grains).

In **Figure 19**, enumeration data were plotted against lithology throughout the core. Lower abundances are associated with the lithologies deeper within the crater (e.g. oxidized clay, diamicton and suevite), consistent with the lower cell counts in Zones 1b-c and 3. Porosity measurements show that lower porosities (generally less than 35%, data provided by the research team at the USGS in Reston, VA) are associated with deeper clasts within the crater and lower cell numbers (data not shown). This is in contrast to the post-impact sediments where porosity is greater (>35%), and cell numbers are higher.
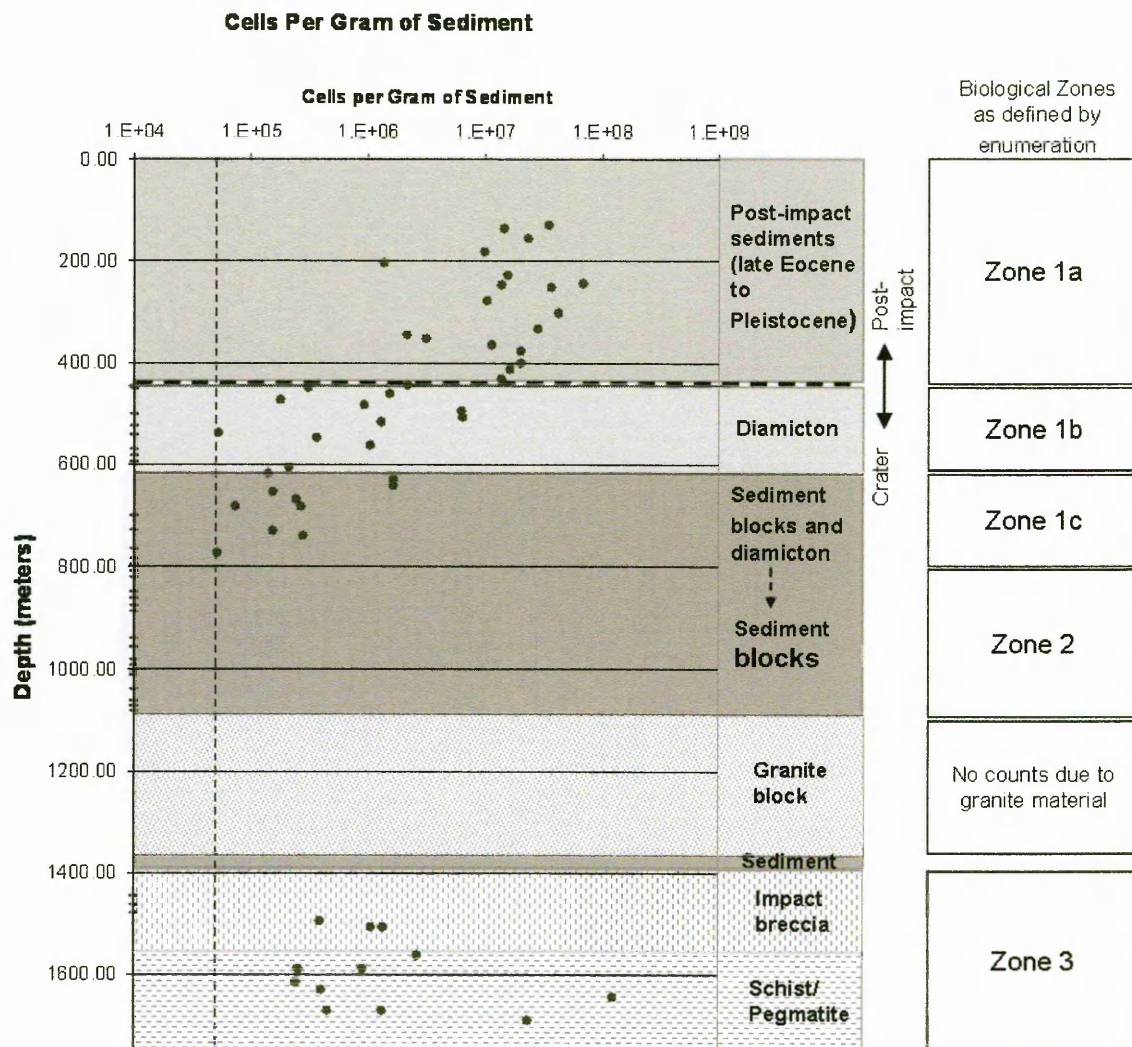
Figure 19 - DAPI enumeration numbers (log abundance per gram dry weight of sediment) throughout the length of the CBIS core. Numbers are plotted alongside general lithology (Gohn 2006). Microbial Zones based on cell numbers are indicated. Depths where samples were collected but where counts fell below the detection limit are indicated by a black dash on the depth axis. Samples could not be retrieved from the granite block because the granite was too hard to crack open for subsampling.

A linear regression analysis of the data above the granite block was performed by Jens Kallmeyer of the University of Bristol in order to compare the decline of cell numbers to those observed at depth in marine sediments. Counts that fell below the detection limit were excluded from this analysis because sediments at these depths did not contain high enough cell numbers to obtain a reliable count. Data from the regression analysis that is specific to the CBIS is shown in **Figure 20**. Although a general trend toward lower cell numbers can be seen, there is a significant amount of scatter in the data points obtained from CBIS counts. Linear regression analysis resulted in a steep slope of the best fit line of

decrease at -3.6. However, the correlation coefficient is low (0.53), meaning that the results of the regression analysis must be treated with some caution.
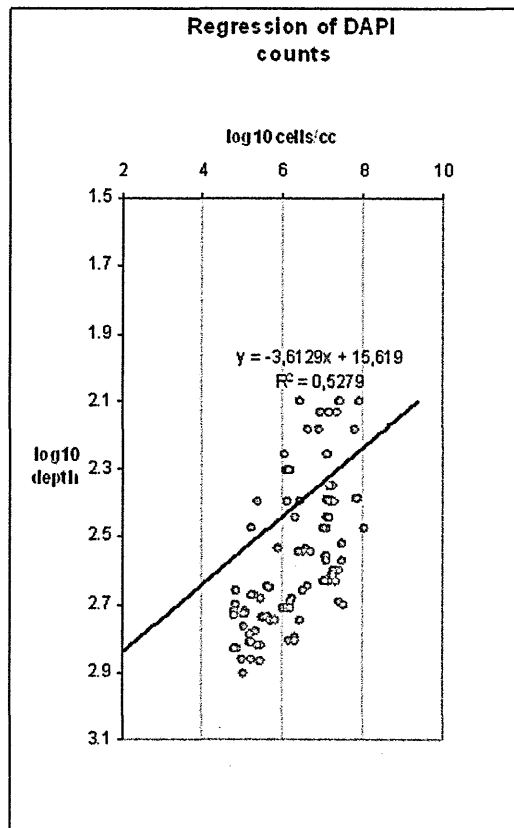
Figure 20 - Linear regression of cell counts from the CBIS above the granite block. Cell numbers and depth are presented on logarithmic scales (data from personal communications with Jens Kallmeyer).

Zone 1

The region dubbed 'Microbiological Zone 1', extending from 127 to 867 m, can be divided into three subsections. Zone 1a (127 to 444) consists of the post-impact deposits of marine, fine-grained siliciclastic sediments that regularly contain biogenic components like shells and microfossils. Data concerning the porosity of Zone 1a sediments was provided by researchers at the USGS in Reston, VA, and is on average between 40 and 55%. Porewater from Zone 1a contains concentrations of DOC and sulfate that are 64 mg/l and 2809 mg/l respectively; and negligible Fe (III) and 1.6 μmol/g Fe (II) (Sanford *et al.*, *In Press*). Zone 1a extends through the post-impact deposits to, or potentially just beyond, the transition into the impact structure, which is thought to rest at ~ 442 m.

Zone 1b (444-618 m) consists of sediment-clast breccias composed primarily of non-marine sediment clasts in a glauconitic matrix. Glauconite is a mica group mineral that

typically indicates continental shelf marine deposits that have accumulated over a long period of time. It appears green in colour, likely due to the presence of iron, and is typically found in Jurassic and lower Cretaceous deposits (i.e. Dodsen *et al.* 1964). The abundance of glauconite grains in Zone 1b are likely due to the mixing of Cretaceous and Tertiary marine sediments from the upper part of the impact target sediment layer with the non-marine, Cretaceous sediments located below. Zone 1b contains sparse amounts of shocked rock, shocked mineral clasts and melt particles. In general, the sediments of Zone 1b appear more sandy than those of 1a and contain diamicton (i.e. poorly sorted sediment of large grain size).

Zone 1c (618 to 867 m) consists of mostly clasts and matrix derived from non-marine Cretaceous target sediments. Zone 1c is also sandy and rich in glauconite, and sediment blocks with diamicton intercalations. Cell enumerations estimates in Zone 1c are low but above the detection limit.

For Zones 1b and 1c, the average concentrations of DOC and sulfate were 27 mg/l and 1729 mg/l respectively; and Fe (III) was negligible and Fe(II) was 0.49 µmol/g (Sanford *et al.*, *In Press*). The diamicton in Zone 1b and the sediment megablocks containing diamicton intercalations in Zone 1c are thought to have been deposited into the crater by the ocean resurge that occurred after impact. The sandy, glauconite-rich sediments in Zones 1b and 1c are generally unsorted and unstratified.

Zone 1 shows a logarithmic decline in cell numbers from nearly 10^8 down to 10^4 cells per gram (i.e. down to below the detection limit of DAPI Method 2). The decline of cell numbers begins in post impact sediments that were deposited over 35 million years following the formation of the CBIS. This decline continues through the upper section of resurge sediments that were deposited as ocean waters and material flowed back into the impact structure. This decline is qualitatively similar to enumeration studies performed on deep-sea marine sediments (e.g. Parkes *et al.* 1994; D'Hondt *et al.*, 2004). However, the decline seen in CBIS samples is steeper than that previously reported in marine sediments

covering a similar geologic time interval (i.e. sediments ranging from the late Eocene to the present).

A similar decline in cell enumeration estimates was observed by Chapelle *et al.* (1987) in non-contiguous cores collected at a site roughly 25 miles west of the outer rim of the CBIS. The cores were collected in unconsolidated Coastal Plain sediments from within the Patapsco aquifer, which contains fresh water younger than 10,000 years. The water within the aquifer is recharged in or near sediment outcropping or subcrops and flows to the south and east. The sediments obtained by Chapelle *et al.* (1987) are from a geologic zone that covers modern to lower Cretaceous sediments. This is similar to that of the CBIS, but the cores were obtained in a shallow depth of under 200 m. The Chapelle *et al.* (1987) cores contained lignitic organic matter, but showed a very low concentration of Dissolved Organic Carbon (DOC) (i.e. in the range of -0.4-1 mg/l).

The decline in cell numbers observed in the Chapelle *et al.* (1987) study and in studies from deep marine sediments could be due to the low availability of carbon at depth. However, this does not explain the steep decline observed in samples from the CBIS because DOC concentrations in the CBIS significantly exceed those measured in both environments.

DOC concentrations appear to be high throughout the entire length of the CBIS core (i.e. >16 mg/L) (Sanford *et al.*, *In Press*). Total Organic Carbon (TOC), on the other hand, is high only in certain regions of the core. The interval between 200-400 m shows TOC concentrations that can exceed 1.36% by weight, and TOC is at its highest (2.22%) at 1671 m (Sanford *et al.*, *In Press*).

There is no linear correlation between either TOC or DOC concentration and cell numbers, but the high concentration of TOC in the post-impact sediments may indicate greater availability of biologically useful carbon. This region corresponds to higher cell counts observed in microbiological Zone 1 relative to counts from the sediment-clast breccias.

Porosity of the core sediments varies with depth and sediment type, and this could also contribute to changes in cell number estimates. Porosity of the material reflects the amount of occupiable space available for microbes to inhabit. Microbes colonize these pore spaces in sediments, and a higher porosity can effectively provide more room for microbial communities to establish themselves (i.e. Cockell *et al.* 2002, Omelon *et al.* 2006; Walker and Pace 2007). In general, porosity in the CBIS is higher in post-impact sediments (Zone 1a) than in sediment-clast breccias (below Zone 1a). However, the porosity does not show a sequential decline with depth through the post-impact sediments. This indicates that porosity alone is not responsible for the logarithmic decline in cell numbers as depth increases.

Although porosity is high in the post impact sediments (Zone 1a), the hydraulic conductivity is low in some sections. This means that although there may be space available for cells to colonize, there may be insufficient flow of water to help transport non-motile cells and nutrients. A limited supply of nutrients in turn limits cell growth, and the abundance of cells could then be reduced. The sandy, glauconite-rich material of Zone 1b has a lower porosity than sediments from Zone 1a. However the unstratified nature of material in Zones 1b and 1c may allow these regions to become more accessible to microorganisms and nutrients.

Another factor is the concentration of salt observed in CBIS sediments. An increasing gradient of salinity is present through the upper portions of the CBIS core (Zone 1 and Zone 2), and salinity concentrations substantially exceed that of seawater in some sections of the core (Poag, 1999; Sanford et al., 2003; McFarland and Bruce, 2004; 2006). Salinity reaches almost 2 times that of seawater between 800-1100 m (See **Figure 21**). There are some marine organisms that can survive at these salinity levels, and a rare few prefer high salt concentrations. However, most known microorganisms are unable to survive at such high salinity (Grant 2004). High salt concentrations can inhibit microbial

growth, and the high salinities present in the CBIS could limit the diversity and abundance

of microorganisms.
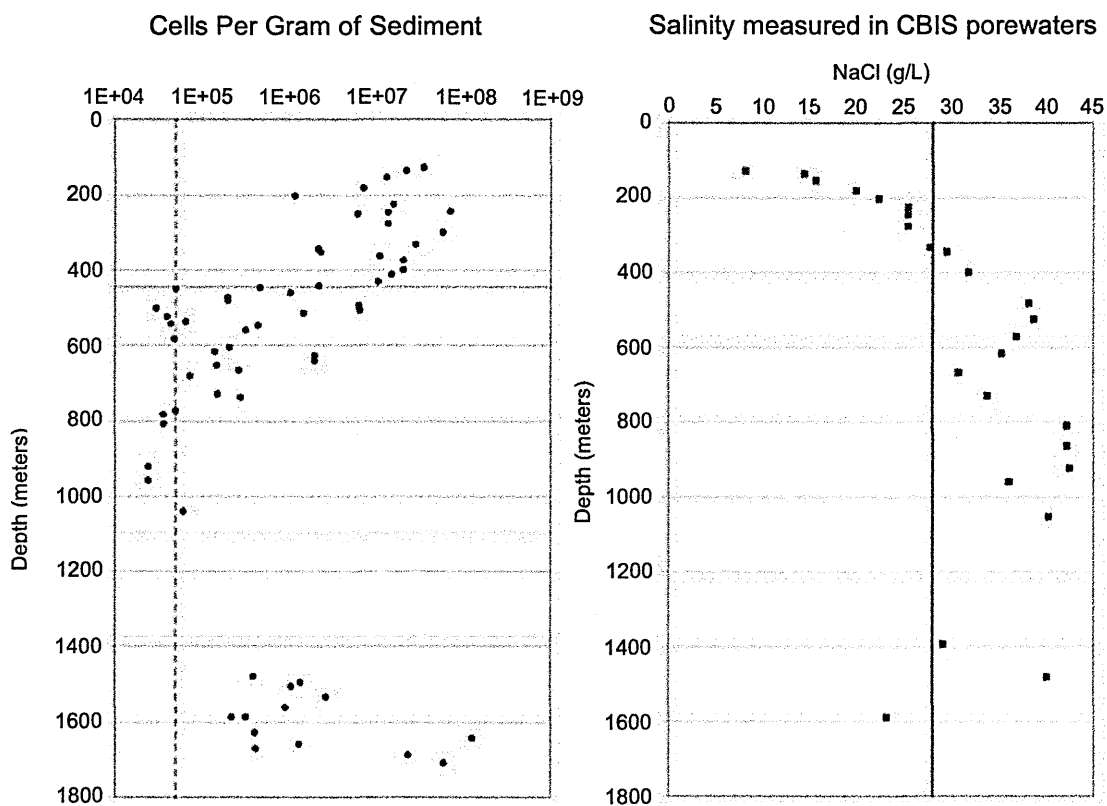
Figure 21 - DAPI enumeration of cell numbers plotted alongside NaCl (g/L) in porewaters drawn from the CBIS. Data on salinity was provided by Ward Sanford at the USGS in Reston, VA. Average NaCl content for seawater (28 g/L) is indicated by a solid, horizontal line plotted against the data for NaCl (g/L) in CBIS porewaters.

Hydrothermal boiling of waters within the CBIS as a result of the thermal heating

of the crater forming impact could have feasibly created the briny liquids present in the

CBIS. However, the volume of brine suggests that it is instead a relic of the pre-impact

environment that was never completely flushed out of the crater due to the low

hydrological conductivity of the sediments (Sanford 2003, 2005).

Small-scale variations can be seen within the trend of logarithmic decline

throughout Zone 1. The variations present in the top sections of the core might reflect the

small-scale changes in mineralogy, sediment grain size and/or *in situ* geochemistry

associated with the post-impact sediments that were deposited at 127-440 m. Cell

abundances do not appear to be directly related to the specific sediment type. Cell numbers

here are higher in clays from the post impact sediments than in clays from the ocean-resurge deposits inside of the crater. The same is true for sand clasts from the two different areas. However, within the post-impact sediments of Zone 1a, sediments containing sandy silt and higher values of TOC show higher cell numbers than the clay-rich clasts with low TOC (Cockell *et al., In Press*; Sanford *et al., In Press*).

In Zones 1b and 1c, the variations in cell numbers are larger than those observed in Zone 1a. This might be the result of abrupt changes in lithology and the combination of very different source impact material resulting from the forces generated during insurge, antisurge and oscillations of ocean resurge into the crater cavity (Powars *et al.*, 2007 and Powers *et al., In Press*). This idea is supported by the differences in mean cell abundance within clasts (Cockell *et al., In Press*). There is likely a great deal of variation in cell numbers at small scales within zones of apparently similar clast definition. Variability in cell numbers at depth have previously been correlated with lithological changes in studies of subsurface environments (Inagaki *et al.* 2003) or changes in interstitial water geochemistry (Parkes et al., 2005, D'Hondt et al. 2004; Biddle et al., 2005).

Zone 2
Microbiological Zone 2, from 867 to 1096 m, corresponds to the lower region of sediment breccias that rest above the granite megablock. Zone 2 is primarily composed of Cretaceous non-marine sands and clays from the lower section of the target sediment layer. These materials are present as intact clasts of up to 17 m in size, and as disaggregated matrix between the clasts. Salinity in Zone 2 peaks at 60 g/l and the average porosity is 20-30%. Concentrations of DOC and sulfate in porewaters are 18 mg/L and 1758 mg/L respectively; and Fe(III) is negligible and Fe (II) is 1.33 μmol/g (Sanford *et al., In Press*).

Cell enumeration estimates in Zone 2 fell below the detection limit of DAPI Method 2. In addition, cell cultivation and molecular biology studies at nearly all depths do not indicate the presence of any cells (see **Chapter 4**). The porosity of Zone 2 is low and porewaters contained within this section of the core are a relic of the immediate post-

impact environment. The absence of cells in Zone 2 is consistent with an impact scenario that includes sterilization of areas in this section by heat generated either directly by the impact or as a result of vertically advected heat from the suevite and impact melt rocks below (Sanford, 2005; Malinconico *et al., In Press*). If this section of the core was sterilized by impact-generated heat, the high salinity present in porewaters may have prevented recolonisation by microorganisms once the temperatures decreased. If this is the case, Zone 2 has remained biologically impoverished since the impact event.

However, geological information on the CBIS core suggests another hypothesis. Zone 2 corresponds to the lowest region of sediments that may have been deposited inside the crater structure as the result of an avalanche from the walls of the crater, along with the granite megablock. This idea is supported by the fact that the sediment sequence of Zone 2 is similar to the target stratigraphy present before impact, where Cretaceous non-marine sediments rest on top of pre-Mesozoic igneous and metamorphic rock. The avalanche that deposited the sediments of Zone 2 may have consisted of multiple slides that rode over one another.

Cell enumeration estimates that lie below the detection limit in Zone 2 could suggest that the material composing this sedimentary sequence contained low microbial numbers prior to impact and was emplaced into the crater as a single lithologic unit. Zone 2 may have remained biologically impoverished following this emplacement despite an upward flux of water from beneath the suevite section. Because of the low hydraulic conductivity of Zone 2 sediment, this upward flux of water may have moved through more conductive material that the emplaced Zone 2 units, leaving the original porewater of Zone 2 intact.

Zone 3

Microbiological Zone 3 (1397 to 1766 m) contains two distinct lithologic units. The first unit (1397-1551 m) is composed of suevitic (melt-bearing) and impact lithic breccias (containing shocked and unshocked clasts) corresponding to ground-surge (i.e. material

from beneath the point of impact that was forced upward but never left the crater) and aerodynamic fall-back material (i.e. materials ejected into the air that then fell back into the crater). The second unit (1551-1766 m) is composed of cataclastically deformed (containing angular fragments formed by fracture and rotation) schist (metamorphic) and pegmatite (coarse-grained igneous rock).

Within Zone 3, the average porosity ranges from 5 to 15%. The average concentrations of DOC and sulfate in porewater are 44 mg/l and 1574 mg/l respectively; and sediment Fe (III) is negligible and Fe (II) is 3.6 μmol/g (Sanford *et al.*, *In Press*).

Zone 3 represents a biologically important section of the CBIS core because a rise in cell numbers is present below the granite megablock in the suevite and fractured schist and pegmatite rock. The materials present in Zone 3 were likely sterilized due to impact-induced hydrothermal activity (Sanford 2005) or by heating from melt-rocks. This means that microorganisms were reintroduced after the ambient temperatures within Zone 3 fell below the upper temperature limit for microbial growth, which is thought to be <121°C (Kashefi and Lovley 2003). Cell numbers rise above the detection limit at ~1480 m and reaches ~10^6 cells per gram sediment at the base of the suevite (melt-bearing) section. Cell numbers remain high in the schist/pegmatite section the suevitic (melt-bearing) and impact lithic breccias (1397-1551 m).

This upper section of Zone 3 contains 20-30% impact-melts by volume (Horton *et al.*, *In Press*). Minerals such as quartz display shock features, such as planer deformation. The degree of shock seen in minerals and the high presence of impact-melts indicate that this upper section, or at least the material it is composed of, was subjected to temperatures during impact that would have been sufficient to sterilize the material. The amount of impact-melts in this section suggests that the average temperature of materials during the time of deposition would have been >350°C, based on numerical calculations used for modelling thermal maturity (Malinconico *et al.*, *In Press*). This, of course, exceeds the known upper limit for microorganism survival (>121°C steam or >160°C dry heat).

Microorganisms would have had to recolonise this section after temperatures fell to below the upper limit for growth. There is no evidence to support regional lateral advection in this region. Microorganisms may have gradually diffused in from nearby clasts that did not experience sterilizing temperatures, or they may have been carried into the section via compaction-driven vertical advection from the permeable schist and pegmatite region located at the bottom of Zone 3 (Sanford *et al.*, *In Press*).

Rocks in the lower part of Zone 3 (1551-1766 m) do not exhibit shock features, but impact-melt particles are present in suevites, and breccia dikes containing shock-deformed clasts. There are also fracture networks associated with the dikes and some narrow veins show networks of micro-fractures. These features indicate that brittle cataclastic deformation and fracture formation occurred during or after the impact event. The lack of shock features in the rocks is inconsistent with their proximity to the crater centre. This likely indicates that the lower region of Zone 3 is not *in-situ* basement rock. Instead, this material likely slumped into position from the transient cavity in which fracture formation was induced by the attenuated shock wave caused by the impact.

There is no corresponding enumeration data available for unaltered materials at this depth from outside of the crater. However, Chapelle *et al.* (1987) obtained enumeration estimated of 10^3 cells per gram in similar materials at far more shallow depths, but corresponding to Cretaceous sediments.

The CBIS enumeration data suggests that microorganisms would have migrated into this region of the crater to colonize new habitats created as a result of impact and post-impact processes that served to increase the permeability of this region through the creation of fracture networks. The presence of breccia dikes in this region suggests that dilatancy, or fracture opening, occurred during dike emplacement before sediments were emplaced above. This would have contributed to an increase in permeability for later recolonisation by microorganisms.

3.8 Summary

The results of cell enumeration estimates throughout the length of the CBIS core reveal patterns of abundance that correspond to distinct lithological transitions within the crater. The logarithmic decline of cell numbers is consistent with trends in other deep subsurface environments. However, the gradient of decline is more pronounced when compared to deep sea marine sediments. The increases in cell numbers observed in the deepest section of the core indicate that recolonisation occurred at some point following impact. This process of recolonisation was likely facilitated by impact-induced fracturing of the target materials and post-impact compaction-driven vertical advection. This study, for the first time, indicates that even if impact processes can potentially sterilize materials through the force of impact and thermal heating, impacts can also generate new habitats for subsequent microbial colonization.

Chapter 4: Culturing of Microbes in the CBIS

4.1 ABSTRACT

One of the primary goals of this study was to obtain cultureable organisms from CBIS core sediments. It was expected that such a large, convulsive impact event would have a noticeable and lasting effect on subsurface microbial communities. As target materials are altered by impact, the accessible microenvironments and availability of minerals and nutrients will be altered as well; which will be reflected in the microbial communities that these microenvironments support.

Attempts to culture organisms were made using CBIS sediment samples at varying depths throughout the length of the core. Media for culturing heterotrophic, iron reducing, sulfate reducing, humic acid utilizing, methanogenic and iron oxidizing microorganisms were used to obtain enrichment cultures in liquid media and on solid media. Serial dilutions on plates were attempted to obtain isolates from each successful enrichment culture. Most probable number estimates were attempted on a selection of heterotrophic enrichment cultures to obtain estimates of the original biomass present in sediment samples.

The results of culturing studies show that cultureable organisms are present in the upper regions of the CBIS core corresponding to post impact sediments, and down through the transition into resurge breccia. A region where few or no cultureable organisms could be obtained was found in lower region of sediment breccias that rest above the granite megablock. This corresponds to the microbiological 'Zone 2' defined by enumeration studies (See **Chapter 3**), where enumeration estimates fell below the level of detection. Cultures were obtained from the deepest core sediments, indicating that re-colonization of this region must have occurred at some point after thermal heating generated by the impact had subsided.

Culture studies support the conclusion that while the thermal pulse generated by impact events may serve to sterilise subsurface sediments, impact-induced fracturing and restructuring of target materials can generate new habitats for microbial colonization.

4.2 MICROBES IN THE DEEP SUBSURFACE

Following the identification of microbial cells in CBIS cores using direct counts for enumeration (**Chapter 3**), the next step in examining the microbial biosphere of the CBIS was to culture living microorganisms from core materials. Obtaining microbial cultures was a key focus in defining the subsurface microbial ecology of the CBIS. When drilling began in September of 2005, the subsurface environment of the CBIS at the drill site was wholly uncharacterized. There was no prior knowledge of sediment types or lithologic structure of the core specific to the drill site; and the microbial community, if present at all, was entirely unknown. A generalized approach was therefore taken in obtaining microbial cultures from CBIS cores, in which as many different media types as possible were inoculated with CBIS sediments in an effort to obtain a variety of organisms that could then be used to better understand the deep microbial biosphere at the CBIS site.

Although the specifics of the subsurface environment at the CBIS site were unknown prior to drilling, subsurface communities were not expected to be drastically different than those observed in studies on other subsurface environments. Generally speaking, the material that composes subsurface environments contains multiple pore spaces and microsites inhabited by microorganisms. These microsites can be vastly different across small spatial scales in terms of important inputs, such as nutrient availability, for microbial growth. Microsites in the subsurface typically present a mixture of anaerobic and aerobic environments, meaning that both anaerobic and aerobic organisms can be cultured from sediments (Amy and Haldeman 1997, p 148). In most subsurface environments, groundwater recharge tends to provide an influx of dissolved oxygen, allowing for the growth of subsurface aerobes (Amy and Haldeman 1997, p 148). The activity of these organisms then reduces the concentration of O_2 in the environment.

Aerobic heterotrophs are the most commonly studied subsurface organisms because these organisms are dominant in the shallow subsurface environments, which are easier to sample than deep subsurface sediments. Aerobic organisms are also easier to study because, unlike anaerobes, their cultivation does not require the use of special equipment to maintain an appropriate oxygen-free atmosphere. The shallow subsurface tends to be aerobic and contains numerous organic compounds that can serve as energy sources (Amy and Haldeman 1997, p 146).

Oxygen is abundant at the Earth's surface and its role in the surface biosphere is immense. When progressing downward through the subsurface, and further from the oxygen reserves of the atmosphere, oxygen becomes depleted and anoxic environments persist. This is not to say that aerobic environments do not exist deep in the subsurface. Dissolved oxygen can be present in groundwater reserves, and numerous aerobic organisms have previously been cultured from subsurface aquifers and sediments (e.g. Balkwill *et al.* 1989; D'Hondt *et al.* 2004; Fredrickson *et al.* 1995; Schmid *et al.* 2007). However, because of the depth at which CBIS cores were recovered and the apparent lack of hydraulic conductivity in sediments, it was expected that if microorganisms were present at all, most microenvironments in the CBIS were likely to support anaerobic communities (See Chapter 1 for a description of hydraulic conductivity in the CBIS).

The general trend in subsurface environments is for conditions to become more and more anoxic with depth as distance from the surface biosphere, and the oxygen-rich atmosphere, increases. As O_2 becomes depleted in the environment, other pathways for respiration begin to dominate. The typical pattern goes from aerobic respiration to nitrate and manganese reduction, Fe(III) reduction, sulfate reduction and methanogenesis (Lovley and Chapelle 1995). This spatial pattern of metabolic pathways occurs in many environments where oxygen becomes depleted over distance from a source, including aqueous environments (lakes, oceans, etc.) and in biofilms (Amy and Haldeman 1997, p 148). In aqueous environments and biofilms the distribution of metabolic pathways can

occur on scales of less than a centimetre. In the subsurface, this gradient is often spread over much larger distances.

For subsurface environments, a predictable metabolic gradient is not only present from the surface downward, but can also form around concentrations or pockets of organic matter in the subsurface (i.e. dead organic material, spilled organic pollutants, etc.). In such cases, methanogenesis dominates the strongly reduced region immediately surrounding the source of organic carbon and the gradient progresses to aerobic organisms in the oxic zone furthest out (Amy and Heldeman 1997, p 148).

The micro-environments present in the subsurface contain a mixture of viable, dead and moribund cells and can be divided into two general categories based on their preferred growth substrate. Some subsurface microorganisms grow as free cells in porewaters that fill the microscopic spaces between rocks and sediment particles. These organisms derive nutrients and carbon from sources present in the porewater. The second set of subsurface microorganisms directly associate with rocks and sediments and grow attached to particulate matter. These microbes are thought to derive some of their nutrients and carbon from mineral sources in sediments. Porewater also serves to carry nutrients required for growth for microorganisms associated with sediments.

Many previous studies indicate that a higher number of viable and more metabolically active microbial cells are present in porewater than for those attached to sediments (Amy and Haldeman 1997, p 145). Not all studies agree with this conclusion (i.e. Pedersen and Ekendahl 1990), however it is apparent that although attached and free-living microorganisms may have similar nutrient inputs, the community structures and behaviours of these two groups can be vastly different despite their close proximity (Amy et al. 1992).

In general however, microorganisms in the deep subsurface are much less metabolically active and their growth is much more limited than their counterparts at and near the Earth's surface. Microbial growth in the subsurface is limited by both chemical

and physical factors. The generally low percentage of viable organisms at depth is typically associated with a lower nutrient flux than that found in surface environments (Amy and Haldeman 1997, p 146; Kieft *et al.* 1993). In fact, some studies indicate that the majority of microbial cells present in the subsurface are inactive, moribund or dead (Amy *et al.* 1992; Keift and Rosacker 1991; Keift *et al.* 1997).

Microbial communities in the subsurface exist as two general types, heterotrophic and lithotrophic. Heterotrophic subsurface microorganisms are consumers and require organic carbon deposited in sediments or transported through groundwater (or the migration of petroleum) in order to be metabolically active. The availability of organic carbon typically results from the migration of carbon sources from the surface to the subsurface, meaning that the majority of heterotrophs are in some way tied to the surface biosphere. The term lithotrophy literally means 'rock consumer'. Lithotrophic subsurface microorganisms can utilize inorganic geochemical nutrient sources (typically minerals) for microbial growth (Amy and Haldeman 1997, p 210). Chemolithoautotrophs use the oxidation of inorganic compounds to produce energy. Unlike photosynthesis, chemolithotrophy requires no input of solar radiation for energy production (Jannasch 1995). Ultimately, lithotrophic microorganisms are able to convert inorganic carbon sources to organic carbon, using only inorganic compounds as electron donors and acceptors for energy.

Both heterotrophic and lithotrophic organisms in the subsurface tend to be rather slow growing due to the limited supply of carbon sources and nutrients. Heterotrophs can survive in subsurface environments as long as sources of organic carbon and nutrients last, and in the presence of organic matter, heterotrophic communities tend to be limited by the diffusion and transport of electron acceptors through groundwater (Amy and Haldeman 1997, p 211). Sedimentary formations, for instance those formed by the settling of layers of marine particulate matter including dead marine organisms, tend to be rich in organics.

4.2.1 Subsurface Sediments of the CBIS

The Chesapeake Bay Impact Structure contains numerous mixed lithologies and impact-altered materials. However, the environment of the CBIS should be able to support microbial growth, both heterotrophic and lithotrophic, as long as the necessary substrates are available for energy production and nutrient availability is not limiting. The upper region of the CBIS core consists of sedimentary deposits from the late Eocene, and would be expected to support microbial growth similar to other sedimentary formations found on the Atlantic coastal plain (See **Chapter 1**).

The subsurface of the Atlantic Coastal Plain, on the whole, is thought to have been created by cyclic deposition and erosion of sediments over geologic periods. This passive deposition of sediments left orderly layers that were modified only occasionally by Appalachian tectonism, paleoclimate change and isostatic adjustments (Poag 1997). The impact that created the CBIS caused a massive disruption in this orderly system of layered sediments, mixing the lithologies, and potentially re-distributing important nutrients and carbon sources at varying depths (See **Chapter 1**). The affects of the CBIS impact are thought to extend down to Proterozoic (2.5 billion years ago to 543 million years ago) and Palaeozoic (543 million years ago to 248 million years ago) crystalline basement rocks (Poag 1997).

Most subsurface organisms studied thus far survive in sedimentary habitats (Amy and Haldeman 1997, p 210). These organic-rich environments allow for growth of microbial populations until resources have been depleted. Typically, as you travel deeper into the subsurface and into older and older sediments, the availability of important carbon sources and nutrients is decreased because, over time, microorganisms have depleted these important caches for life. As this depletion of resources occurs, patchy distributions of microbial populations survive around smaller sources of nutrients until the populations, along with the nutrients, become extinct (Amy and Haldeman 1997, p. 211).

Before the occurrence of the CBIS-forming impact, one would expect that this pattern would exist in the Atlantic Coastal Plain on the eastern shore of Virginia. However, the impact excavated materials spanning from the Proterozoic to the Eocene, altered and mixed them and then re-deposited them into the crater along with numerous other materials present in the tsunami resurge (See **Chapter 1**). The effects that this restructuring and introduction of material had on the microbial populations in the CBIS are entirely unknown. It is possible that new sources of organic carbon, nutrients and minerals were redistributed, providing new habitats in sediments that would otherwise have been depleted of nutrients. Additionally, large impact craters have been shown to be sources of precious metals, and metal-rich regions would be expected to be present in the inner basin of the crater (Poag 1997). Such resources may serve as potential sources for redox couples used to power specialist microbial communities.

A second consideration is the idea that the CBIS-generating impact produced a thermal pulse sufficient to sterilize sediments in the CBIS (See **Chapter 1** and **3** for additional information). Although sediments were excavated from the crater structure and then mixed back into the crater along with tsunami resurge materials, the sufficiently sterilizing thermal heat generated by the impact may have persisted for significant periods of time (Sanford 2003). This means that sediments within the CBIS would have been sterilized and remained sterile after impact. The low amount of lateral groundwater flow as evidence by the inland salt-water wedge (See **Chapter 1**), would also indicate that influx of microorganisms after sterilization would have been low. It is possible that microorganisms have not had an opportunity to re-colonize. The sediments of the CBIS may remain sterile to this day.

It should be mentioned that the CBIS might support a range of oligotrophic organisms. These organisms are those that grow in environments with very low carbon concentrations, and may actually be unable to grow in the presence of high nutrients (Koch 2001; Poindexter 1981; Schut et al. 1997). Oligotrophic organisms are characterised as

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having low metabolic rates and are very slow growing even when growth conditions are thought to be optimal (Kock 2001). Such organisms could be present in the CBIS at depths where nutrient inputs and carbon sources are limited, although it should be noted that, based on data from the USGS (Figure X), sources of carbon appear to be high through the depth of the core.

Early attempts to culture organisms from the CBIS using limited amounts of carbon and nutrients were made. However, because oligotrophic organisms can take very long periods of time to grow in culture, it was decided that such organisms would likely be impossible to obtain and examine within the time constraints of this PhD project. In fact, the cultureable organisms obtained from CBIS core samples were obtained with concentrations of nutrients and carbon sources that were likely much higher than those present in the natural environment. In general, the organisms that are most likely to grow in culture are those that are fast-growing and can quickly access the nutrients available.

Culturing microorganisms from CBIS sediments was used to determine whether or not CBIS sediments represent a habitat that currently supports microbial communities. At depths where microorganisms could not be cultured, tests were performed to see if the sediments themselves were incapable of supporting growth, or if it was possible that cultureable microorganisms had simply been unable to re-colonize these sediments following sterilization due to the thermal impulse generated by the impact. The presence of organisms helped to determine how restructuring through impact processes affects the habitability of the subsurface biosphere.

4.2.2 Potential Microbial Environments of the CBIS

The known presence of highly saline groundwater in the region associated with the crater structure and the presence of Eocene marine sediments above the crater structure suggested that microbial studies on seafloor sediments could be the most appropriate analogue for culturing tests. Previous studies by the Ocean Drilling Program on microorganisms at

depths of up to 800 m below the sea floor guided many of the methods implemented in the CBIS study (D'Hondt *et al.* 2004).

Handling and storage of samples has been shown to greatly affect microorganisms, typically causing an increase in microbial activities (Brockman *et al.* 1992). The process of obtaining samples can cause momentary changes in temperature, oxygen exposure and many other factors that vary from the natural environment of sediments at depth. These changes can be detrimental for some organisms and beneficial for the growth of others, sometimes causing dramatic changes in the composition of microbial communities present (Amy and Haldeman 1997, p 146). In order to obtain cultureable organisms, it is important to avoid long-term storage of samples and to inoculate media as soon as possible after sample collection. Because core samples for culturing studies were collected at the same time as those for geological analysis, culturing studies were begun before the subsurface lithologies and elemental composition of the CBIS cores were determined. As data concerning the structure of potential habitats in the CBIS (i.e. lithologies, mineral composition, salinity, pH, etc.) became available, more focused attempts were made to culture specialist organisms at specific depths according to the availability of redox couples (i.e. Fe(II)/Fe(III), sulphate/sulfide etc.).

4.3 METHODS

Media types adapted from D'Hondt *et al.* (2003) for a variety of culture types were chosen as a starting point from which to examine the cultureability of microorganisms present in CBIS samples. Generalized media for heterotrophic organisms were prepared and inoculated with CBIS sediment. Media for chemolithoautotrophic organisms was also prepared and tested at multiple depths. A total of 14 media were inoculated with CBIS sediments, including: two general heterotrophic media, one humic acid utiliser media, three iron reducer media, five sulfate reducer media, two methanogen media and one iron oxidizer media. All of these media were prepared anaerobically in liquid form, and a select few were also prepared aerobically and as solid media in Petri dishes.

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4.3.1 Collection of Samples

Core samples used for culturing studies were collected throughout the length of the CBIS core. Samples were collected on average every 29.3 meters, with the largest distance between sampling being 109.7 m and the shortest being 1.5 meters (**Table 6**). As with the enumeration samples, culturing samples were not collected from the granite megablock from 1094.14 m to 1391.9 m because the solid material was too difficult to crack open and subsample.

Table 6 Sample depths for culturing studies throughout the CBIS core.

Sample Depth (m)	Sample Number	Distance Between Samples (m)	Sample Depth (m)	Sample Number	Distance Between Samples (m)
126.9	1	N/A	844.6	26	2.5
159.3	2	32.4	899.2	27	54.6
241.5	3, 4	82.2	901.0	30	1.8
250.5	5	9.0	941.9	31	41.0
360.2	6, 7	109.7	970.3	32	28.4
366.6	8	6.5	997.7	33	27.4
435.6	9	68.9	1020.2	34	22.5
441.7	10	6.1	1047.7	35	27.5
447.0	11	5.3	1085.5	36	37.7
449.3	12	2.3	Granite Megablock 1094.1		
500.8	13	51.5	1391.9	37	N/A
510.6	14	9.8	1419.1	38	27.1
544.3	15	33.7	1447.5	39	28.4
571.1	16	26.8	1473.3	40	25.8
588.1	17	17.0	1509.4	41	36.1
614.6	18	26.5	1537.4	42	28.0
643.5	19	28.9	1562.4	43	25.0
677.8	20	34.3	1567.1	44	4.7
702.5	21	24.8	1568.5	45	1.5
744.6	22	42.1	1608.8	46	40.2
770.8	23	26.2	1642.5	47	33.8
787.4	24	16.6	1677.4	48	34.9
821.0	25	33.7	1740.4	49	63.0
842.1	28, 29	21.1	1752.6	50	12.1
			Average Distance Between Samples		29.3

Subsections of CBIS cores for culturing studies were collected on site at the Eyreville Farm immediately upon core retrieval. For a description of the drill site, see **Chapter 1**. Core sections of roughly 0.1 m were selected prior to washing of the core for geological studies. When possible, the outermost rind was pared away and the subsection was then moved to a UV-sterilized laminar-flow hood in the on-site microbiology laboratory. Between sampling, all workspaces were cleaned with ethanol and instruments were autoclaved and/or flame-sterilized. All core samples were handled with latex gloves

on pre-autoclaved pieces of sterile foil. For each step in handling samples, the core was transferred by use of sterile tongs to a fresh piece of sterile foil.

Samples of the exterior and interior of core subsections were collected and tested for indicators of contamination (see **Chapter 2**). The procedure for handling core subsamples can be found in **Chapter 2**. Un-split core subsections were placed in sterile Whirl-Pak® sampling bags (eNASCO) and closed. The Whirl-Pak® was then placed inside an airtight, heat-sealed wine bag along with an anaerobic atmospheric generator sachet to maintain anaerobic conditions. Packaged samples were stored at -5°C and shipped to the Open University, Milton Keynes, UK, where culturing studies were performed.

Samples were then handled in the anaerobic lab in the EGL Laboratories at the Open University. Samples were transferred to the anaerobic chamber (miniMacs Anaerobic Workstation, Don Whitley Scientific, West Yorkshire, England), where wine bags could be opened in anaerobic conditions. The anaerobic chamber was pressurized with an anaerobic gas mixture containing 10% CO_2, 10% H_2 and 80% N_2 (AirProducts Ltd.). Access to the chamber was via N_2-flushed, rubber sealed glove sleeves. Oxygen inside the chamber was monitored in parts per million (ppm) using an oxygen detector (Coy Laboratory Products Inc.) placed inside of the chamber.

All workspaces inside the anaerobic chamber were cleaned with ethanol prior to core handling. Core subsections were placed inside the anaerobic chamber on pre-autoclaved pieces of sterile foil and handled using nitrile gloves using pre-autoclaved and ethanol-sterilized tools. Cores were carefully split open and sediment was collected from the newly-exposed, central area of the core. Sediment was removed from the interior of the core and placed in pre-autoclaved, N_2-gassed rubber stopper glass bottles. When not in use, collected sediments were then refrigerated at 5°C for storage.

To inoculate culture media, core sediments were carefully removed from the glass bottles inside the anaerobic chamber using pre-autoclaved, sterile spatulas. Sediment was

transferred into liquid media and culture bottles were then sealed before removal from the anaerobic chamber. Glass bottles containing the collected sediments were also sealed within the anaerobic chamber, and then gassed with N_2 before being placed back in refrigeration for storage.

4.3.2 Anaerobic Techniques

All attempts to culture microorganisms from the CBIS in this study were performed under anaerobic conditions. Anaerobic organisms are those that do not require oxygen for growth, and produce energy inside their cells by processes such as fermentation. Strict anaerobes are incapable of growth in the presence of oxygen. Over 80 strictly anaerobic strains of microorganisms have been identified (Amy and Haldeman 1997, p 112), and these organisms represent a unique and somewhat poorly understood component of the deep subsurface biosphere. Other anaerobic organisms can also use additional means of energy production aside from fermentation in the presence of oxygen (such as photosynthesis), and are referred to as facultative anaerobes.

To avoid oxygen exposure, all inoculations of liquid and solid media were performed inside the anaerobic chamber. The majority of liquid cultures were stored in metal-capped anaerobic bottles that could be sealed and removed from the anaerobic chamber and stored in incubators (**Figure 22**). When aliquots of cultures were removed from these bottles, pre-autoclaved sterile glass beads were added in order to lift the level of the liquid and remove any air-filled headspace inside the bottle. In some cases, Hungate tubes sealed with rubber stoppers were used to collect isolates and to perform growth tests where smaller volumes of media were required (**Figure 23**). Plate cultures were spread and stored inside the anaerobic chamber at 37°C; near the ambient temperature of sediments at depth in the CBIS. For long-term storage, some cultures were transferred to sealable anaerobic canisters with an anaerobic atmospheric generator sachet to maintain anaerobic conditions. Canisters were then stored alongside culture bottles in incubators at 37°C.

Figure 22 - Silver capped anaerobic bottles used for culturing experiments.

Figure 23 - Hungate tubes with rubber stoppers used for culturing experiments.

4.3.3 Heterotrophic Enrichments

Heterotrophic organisms require organic substrates from which they derive energy for growth. Heterotrophic microorganisms can utilize a wide range of organic matter, and the majority of known microorganisms present in the biosphere fall into this classification. In order to obtain energy, many heterotrophs (aside from fermenters) can also utilize a wide range of electron donors and acceptors, meaning that some heterotrophs can fall into microbial categories such as 'iron reducers' if they require carbon from organic sources for growth and the reduction of iron minerals for energy. The breakdown of organic compounds in anaerobic conditions often requires collaborations between consortiums of microorganisms that utilize different electron acceptors in the environment. Common electron acceptors in anaerobic subsurface environments include nitrate, nitrite, manganese (IV), Fe (III) sulfate, sulfur or CO_2 (Amy and Haldeman 1997, p 113).

Two general heterotrophic media were used to obtain heterotrophic enrichment cultures from various depths within the CBIS. These media contain a wide range of carbon sources and are designed to promote the growth of a similarly wide range of heterotrophic species. Heterotrophs obtained with these types of media tend to be the easiest to grow in laboratory conditions because they are generalists with growth requirements that are typically less specific than specialist microorganisms.

The two general heterotrophic media selected for culturing organisms from CBIS core samples are referred to as Heterotrophic Media DH (Het Med DH) and Heterotrophic Media 1 (Het Med 1) (See **Appendix II**). Het Med DH was adapted from a media used by D'Hondt *et al.* (2003) and Süßa *et al.* (2006) for marine sediments. Het Med 1 was adapted from Stevens *et al.* (1995) and Tsai *et al.* (1995) for use with samples from a deep terrestrial site in Washington State, USA. Het Med DH was prepared anaerobically under N_2 gas. Het Med 1 was prepared aerobically and then oxygen was removed using a reducing agent. Both Het Med DH and Het Med 1 contain various nutrient sources that can provide for the growth of generalist organisms that may be present in CBIS cores.

Oxygen was removed from Het Med 1 by the addition of Na_2S (**Appendix II**), and media was immediately transferred to pre-autoclaved, metal-capped anaerobic culture bottles after preparation. The addition of Na_2S causes the formation of a black precipitate as Na_2S reacts with oxygen. Media was added to bottles inside the laminar flow hood to prevent contamination by airborne organisms. Controls were prepared for all replicate inoculations by keeping un-inoculated bottles from every media preparation. Controls were examined for microbial growth at the same time as inoculated samples.

In some experiments, Na_2S was either removed from the media or titrated into the solution in small amounts to achieve anaerobic conditions. This is because the black precipitate produced by the reaction of Na_2S with oxygen made it more difficult to identify cells growing in solution in some experiments. To achieve anaerobic conditions without Na_2S, Het Med 1 was gassed with N_2 both before and after autoclaving. The media was

gassed until clear (no colour change shown by oxidation of resazurin), indicating that the media was free of oxygen. If the media had returned to a slightly pink hue before inoculation, small amounts of Na_2S were titrated in until the media went clear.

For the preparation of plates, 2% analytical grade bacterial agar was added to the media prior to autoclaving. Selenite/tungstate, vitamin solution and Na_2S were added once the media had cooled enough for the glass bottle to be handled with nitrile gloves, and media was then poured into Petri dishes inside the anaerobic hood to avoid oxidation of the media as plates cooled and solidified.

Soft top agar plates were prepared with Het Med 1 by preparing two complete media solutions, one containing 2% agar and a second containing only 0.2 % agar. Plates were poured half full inside the anaerobic hood using the 2% agar solution. Once solidified, the second 0.2% of agar was added.

4.3.4 Humic Acid Utilisers

Humic acid is an important component of soil organic matter. Humic substances are formed by the biodegredation of living tissues and biomolecules, and are the precursors for many fossil fuels. These high-molecular-weight organics are resistant to microbial degradation, yet some microorganisms in sediments are capable of utilizing humic for respiration (Lovley *et al.* 1996). These anaerobic microorganisms are able to use humic acid as an electron acceptor for the oxidation of hydrogen and organic compounds, with electron transport yielding energy for growth. Humic acid utilisers may play an important role in subsurface communities by stimulating the oxidation of other electron acceptors, such as Fe in sediments. When humic acids are reduced by microbial action, humic substances can then act to shuttle electrons between humic-reducing microorganisms and metal oxides (Lovley *et al.* 1996).

Media for humic acid utilisers, dubbed HA Med, was mixed at the USGS prior to collection of core subsamples (see **Appendix II**). HA Med was prepared in pre-autoclaved sterile glass bottles sealed with a rubber stopper and metal crimp at the U.S. Geological

Survey in Reston, Virginia. When subsamples of CBIS cores were collected in the onsite microbiology laboratory, HA Med was inoculated and then transferred back to the USGS for storage and incubation. Media was inoculated within a small depth range of samples that included the depths 677.8 m, 702.6 m, 744.6 m, 770.8 m, 787.4 m, 821.1 m, 901.0 m and 1447.5 m.

4.3.5 Iron Reducers

Iron reducers are able to use ferric iron (i.e., Fe(III), Fe^{3+}) as an electron acceptor for the oxidation of organic compounds to CO_2 (Gorby and Lovley 1991). In this form of respiration, Fe(III) is reduced to Fe(II), and energy for growth is ultimately produced. In environments where methanogenesis is not present, and where electron acceptors such as sulfate are present, microbial respiration by iron reduction can be very important (Ehrlich 1996, p363). Iron reduction in subsurface environments is often identified by an increase in ferrous iron (i.e., Fe (II), Fe^{2+}), which accumulates as iron reducing microorganisms reduce ferric iron (i.e., Fe (III), Fe^{3+}) (i.e. Park *et al.* 2006).

Previous studies in the Taylorsville Triassic Basin in northeastern Virginia (outside the northwestern rim of the CBIS) revealed the iron-reducing bacterium *Bacillus infernus* to be the primary iron reducer active at depths from 2.65 km to 2.77 km (Boone *et al.* 1995). The Taylorsville study was conducted at the Thorn Hill 1 drill site near the centre of the Taylorsville Basin, an extensional basin that rests almost entirely beneath the Atlantic coastal plain of Virginia. Sediments from the Taylorsville Basin are not directly comparable to CBIS sediments, as they have not been altered by impact. However, the Taylorsville study can provide some insight into what the environment of the CBIS may have looked like before impact, and the types of organisms present in nearby environments that could re-colonize CBIS sediments if a point of entry is available.

Studies of subsurface environments suggest that iron reduction should be possible in any environment with a source of organic carbon and ferric iron (i.e. Fe(III), Fe^{3+}). For this reason, a selection of depths was tested using iron reducer media. Data on ferric iron

content of sediments was not immediately available after collection of samples. Therefore, depths for the first set of tests for iron reducers had to be selected based on other criteria. Core samples that appeared reddish in colour at the time of collection were noted as this reddish colour may be attributed to iron content. These samples were then used for initial inoculations.

Three media were used for culturing iron reducers. The first, dubbed IR Med DH, was adapted from a media used with seafloor sediments by D'Hondt *et al.* (2003) (**Appendix II**). A second media, dubbed IR Citrate media, was prepared by adding a source of Fe(III) (Iron Citrate) to the standard heterotrophic media, Het Med DH (above). The third media, dubbed IR USGS, was prepared at the U.S. Geological Survey in Reston Virginia prior to sampling. As with the humic acid media, IR USGS media was made in pre-autoclaved sterile glass bottles sealed with a rubber stopper and metal crimp. As CBIS core samples were collected, a small amount of sediment from the interior of cores was added to IR USGS medium during the subsampling procedure in the on-site microbiology laboratory. Samples were then transferred to the USGS facilities in Reston for storage and incubation.

IR USGS media contains a reddish precipitate that darkens when cultures actively reduce Fe(III). In this way, successful cultures were visually identified and then transferred to the microbiology laboratory at the Open University in Milton Keynes (UK) for further study. Cultures were examined under epifluorescence microscopy to confirm the presence of microbial cells.

In addition to liquid cultures, IR USGS media was prepared as solid plates. A 10 mg/mL solution of agar was prepared and autoclaved for 20 minutes at 120°C. As agar cooled, pre-prepared liquid IR USGS media was added (1 mL of agar solution to 5 mL IR USGS media). The solution was briefly mixed by inversion and then poured into sterile Petri dishes under an anaerobic atmosphere.

4.3.6 Sulfate Reducers

Sulfate reducing microorganisms are those that reduce sulfate (or in some cases other oxidized sulfer-containing compounds) to hydrogen sulfide in anaerobic conditions. Sulfate reduction typically happens as a dissimilatory reaction, meaning that sulfur is not directly incorporated into organic compounds (Drake and Akagi 1978). This process occurs by:

$$SO_4^{2-} + 2CH_2O \rightarrow H_2S + 2HCO^{3-}$$
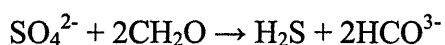
Sulfate reducers can have important repercussions for their local environment. The hydrogen sulfide (H_2S) produced in this reaction then causes the precipitation of metal sulfides, and the subsequent alkaline conditions (HCO^{3-}) produced can act to neutralize acidic waters. These processes are being examined commercially for selectively extracting valuable metals from a substrate and the removal of sulfate and acidity in waters that result from pollution (Kaksonen 2008).

Four media were used for culturing of sulfate reducing microorganisms. The first was adapted from a formula used for heterotrophic sulfate reducers in seafloor sediments by D'Hondt *et al.* (2003), and was dubbed SR DH media. A second, more complex media (SR Med 2) was adapted from Pfennig *et al.* (1998). This media requires preparation of nine separate solutions that are then mixed to produce the final media (**Appendix II**). The third and fourth media (PM1 and PM2) were based on a media for nonsporulating sulfate reducing bacteria by Postgate *et al.* (1963).

4.3.7 Methanogens

Methanogens are microorganisms that produce methane (CH_4), typically by using H_2 to reduce CO_2:

$$4H_2 + CO_2 => CH_4 + 2H_2O$$
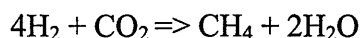
This process is known as biogenic methanogenesis, and is a biochemical process that appears to have evolved early in the history of life (Ehrlich 1996, p 628). Methanogens include a wide range of microorganisms, with a large phylogenetic and morphologic

diversity (Jones *et al.* 1987). Methanogens can be found in nearly all environments where the anaerobic degradation of organic materials occurs, as well as extreme environments like deep-sea hydrothermal vents (Jones *et al.* 1987). The majority of known methanogens are obligate facultative autotrophs, meaning they fix CO_2 to produce organic compounds using inorganic chemicals (typically H_2) as electron donors (Ehrlich 1996, p 631). Additionally, these methanogens can use organic compounds, such as acetate, as electron donors if they are available in the environment.

Two media, dubbed H_2 Methanogen Media (H2 Methano) and Acetate Methanogen Media (A Methano), were used in an attempt to obtain enrichment cultures of methanogenic microorganisms in CBIS cores (**Appendix II**). As with the humic acid media and IR USGS media above, two sets of media for methanogenic microorganisms were prepared at the USGS prior to core sampling. These media were inoculated in the onsite molecular biology laboratory when collecting subsamples from cores and then transferred to the USGS for storage and incubation. Successful cultures for H2 Methano Media and Acetate Methano Media were identified by gas chromatography at the USGS in Reston, Virginia.

4.3.8 Chemolithoautotrophic Microorganisms - Iron Oxidizers

The fourth most abundant element in the Earth's crust is iron, which naturally undergoes redox changes in the environment (Ehrenreich and Widdel 1994). Iron reducing organisms, discussed above, derive energy from the reduction of Fe(III) to Fe(II). The reverse process is also useful for some microorganisms that are able to promote iron oxidation, thereby utilize iron as an energy source for growth (Ehrlich 1996, p. 316). Iron Oxidizing microorganisms obtain energy through the conversion of Fe(II) to Fe(III), using Fe(II) as an electron donor during respiration (Lovley 2000). Fe(II) readily oxidizes to Fe(III) under neutral conditions in the presence of oxygen, meaning that many surface environments are not suitable for iron oxidizers. Fe(II) is stable under acidic conditions or in environments

low in oxygen. Iron oxidizers need to access Fe(II) for energy, and they tend to grow in either acid (pH<5) or anaerobic habitats (Konhauser and Bertola, p. 86).

Acidophilic iron oxidizing microorganisms are often found in geological deposits of metal-sulfides when water and oxygen are present (Lengeler *et al.* 1999). In such habitats, acidophilic iron oxidizers can cause the acidification of the environment. Neutrophilic iron oxidizers are also known to exist, and are commonly found in environments where ferrous iron interacts with oxic environments (Lengeler *et al.* 1999). Novel strains of neutrophilic iron oxidizing bacteria have also been isolated from groundwater environments (James and Ferris 2004).

The role of iron oxidizers in geological processes was recognized in the early 1900s, when enrichment cultures of organisms were used to show lithotrophic growth of Fe-oxidizers (Harder 1919). Interest in iron oxidizing bacteria centres around a number of topics including the early evolution of life, studies of how trace metals cycle in the subsurface and the role of these microorganisms in biofouling and biocorrosion (James and Ferris 2004). Other anaerobic iron oxidizers couple iron oxidation with nitrate reduction in a form of anoxygenic photosynthesis (Lovley 2000). This process was identified in freshwater and marine sediments that were stored anaerobically but exposed to light (Lovley 2000). Some organisms known to oxidize iron are also facultative heterotrophs, meaning that they are able to grow heterotrophically when sources of organic carbon are available, typically using glucose instead of iron as a source of energy and carbon (Ehrlich 1996, p. 316).

4.3.9 Selection of CBIS Cores

Initial culturing tests were performed using Heterotrophic Media DH on sediments within Microbiological Zone 1 as defined by enumeration studies (See Chapter 3). This upper region of the core contained the highest number of microbial cells in epifluorescent direct counts. It was thought that microbial diversity would be highest in these sediments,

increasing the likelihood of obtaining cultureable organisms. Subsequent tests were also performed on these sediments using other media.

Additional culturing tests were then performed using the various media discussed above, and using sediments from depths that ranged throughout the CBIS cores. As data concerning the physical and chemical composition of sediments became available, additional depths were selected in an attempt to culture specific organisms, such as sulfate reducers and iron oxidizers.

4.3.10 Aerobic Tests

Liquid and solid Heterotrophic Media 1, which was the most successful media for obtaining growth from CBIS cores, was also inoculated under aerobic conditions. Although CBIS culture studies were focused on anaerobic microorganisms, it was of interest to determine whether or not aerobic organisms (or possibly facultative anaerobes) were also present in the CBIS cores. Additionally, enrichment cultures that were successfully grown in anaerobic media were tested on plates to see if the organisms were obligate anaerobes or if they could survive in the presence of oxygen.

Heterotrophic Media 1 was made following the procedure outlined in **Appendix II**, except that the reducing agent Na_2S was removed from the formula. Heterotrophic Media 1 was prepared and inoculated aerobically and placed in incubators at 37°C to test for growth. Solid media was made in Petri dishes and spread with 50 μL of anaerobic enrichment culture. Plates were then stored in the aerobic incubators at 37°C.

4.3.11 Impoverished Zone Tests

As discussed in **Chapter 3**, a region of the core where the majority of cell enumeration numbers were below the detectable limit was found between depths 867 meters to 1096 meters, and dubbed Microbiological Zone 2. In this zone, many of the CBIS samples tested yielded no cultureable organisms. In order to determine whether or not sediments in Microbiological Zone 2 are suitable to support life from the deep subsurface, a sediment

sample was selected from this region and inoculated with both enrichment cultures and individual organism isolates obtained from other depths throughout the core. Because cell counts, culture and culture-independent methods showed little or no presence of microbes in this region of the core, it is important to understand if materials from this region present a habitat unsuitable for life. In order to test whether or not crater material from these depths is indeed capable of supporting microbial life, sediment from a depth of 997.7 m was inoculated with microbe cultures obtained from other depths within the core.

4.3.12 Liquid Impoverished Zone Tests

In the first test, liquid Het Med 1 was prepared with all organics removed. To replace the organics needed for microbial growth, 70 mg of sediment from 997.7 m was added to the medium. Although CBIS sediments from 997.7 m showed no growth in previous experiments, the sediment was autoclaved before addition to the medium to ensure that any potential organisms present were removed before addition to the medium. It should be noted that autoclaving could also cause the breakdown of important organics in the sediment that would be required for microbial growth.

The liquid impoverished zone media was inoculated with cultures successfully obtained from depths 571.1 m, 643.5 m, 842.1 m, 1568.5 m and 1608.8 m. After inoculation, the starting concentration of cells in cultures ranged from 1.0×10^1 to 2.5×10^2 cells per mL of culture. Controls were prepared by adding autoclaved sediment from 997.7 m to sterile Het Med 1 (without carbon sources) and no inoculate cultures.

4.3.13 Impoverished Zone Slurry Tests

A second test was prepared by making slurries from CBIS sediments from 997.7 m. Ideally, if sediments from this depth are capable of supporting microbiological growth, they should contain all the necessary nutrients and only the addition of water would be required. Slurries were made by inoculating dry sediment from 997.7 m with growing cultures obtained from other depths within the CBIS. Two types of inoculations were used.

Inoculation 1: Slurries were prepared by adding 200 μL of liquid cultures to 0.5 g of sediment from 997.7 m. Slurries were then incubated at 37°C. Inoculate samples chosen for this test included successful cultures from depths of 571.1 m, 643.5 m, 1568.5 m and 1608.8 m.

As discussed, there is a chance that sediments from 997.7 m contain microbes at levels too low to be detected by DAPI staining and that were simply uncultureable with all techniques attempted. Addition of liquid cultures from other depths could add minerals or nutrients that are required for growth. If this is the case, uncultured microbes in sediments from 997.7 m could then grow and affect the results of this test by producing a false positive. Because of this, a second inoculation was performed.

Inoculation 2: Sediment from 997.7 m was autoclaved at 120°C for 30 minutes to ensure that no viable cells were present. This may have eliminated vitamins and other important organics for growth. However, as mentioned above, negative results for DAPI enumeration and previous culture experiments do not necessarily mean that sediment from 997.7 m is sterile.

0.5 g of autoclaved sediment from 997.7 m was added to 30 mL of Het Med 1 containing no organics. Either 50 μL of liquid culture or a single colony from plate cultures was then resuspended and vials were incubated at 37°C. Culture samples chosen for this test included successful cultures obtained from depths of 614.6 m, 842.1 m and 1608.8 m.

4.3.14 CBIS Media

Attempts to culture organisms from CBIS core material were also performed using well water from the CBIS corehole itself as a growth media. Well water was obtained from the depth of 624 - 626 feet (roughly 190 meters), and will be referred to as Well 626-24. The core at this depth consists of post impact sediments and composed of clay and silt. The total salinity is in the range of 28 to 33 ppt and, based on data from the USGS, the concentration of NaCl is expected to be in the range of 19.8 to 22.3 g/L. Additional

estimates concerning the mineral content of porewater from this depth can be found in

Table 7. Mineral content at this specific depth was not measured, so the numbers shown

are estimates from measurements taken at the nearest surrounding depths. The closest

depth at which direct cell counts were performed was at 126 m. Core samples at this

showed a high number of microbial cells in the range of 3.5×10^7, indicating that sediments

at this depth should be capable of supporting microbial communities.

Table 7 - Mineral Content of Porewater from Depth 624-626 ft. Data provided by the USGS.

pH	7.0 - 7.1
Chloride	12.5 - 14.9 g/L
Ca^{2+}	.58 - .76 g/L
Mg^{2+}	.71 - .8 g/L
Na^+	7.8 - 8.8 g/L
K^+	.37 - .46 g/L
HCO_3^-	.22 - .19 g/L
Cl^-	12.5 - 14.9 g/L
SO_4^{2-}	2.8 - 3.8 g/L
Br-	.048 - .058 g/L
DOC	48.78 - 76.78
Br^-	.048 - .058 g/L

Experiment 1

The first series of experiments were conducted with what was dubbed CBIS 1 Medium.

This medium was made by filtering sterilizing Well 626-24. Resazurin was then added as

an oxygen indicator. Na_2S was titrated into the sterile Well 626-24 until the solution went

clear, indicating that the media was anaerobic. Media was then put in 30 mL anaerobic

culture vials.

Culture vials were inoculated with culture isolates obtained from using Het Med 1.

The cultures selected for the first round of inoculations were from culture depths 571 m,

644 m, 1569 m and 1609 m. Unfortunately, no cultures had been obtained from the depths

at which Well 626-24 was drawn. The cultures used for inoculation were from various

depths in the core and were chosen because these cultures had been shown to be the most

robust in the laboratory and grew the most consistently in previous cross inoculation

cultures using Het Med 1.

Each 30 mL culture vial was inoculated with 150 μL of liquid culture. This volume proved to be sufficient to obtain successful liquid cultures in previous cross-inoculations using Het Med 1. It should be noted that the addition of 150 μL of liquid culture includes not only cells, but also nutrients and other components from Het Med 1. However, diluted in the total volume of 30 mL, this would provide only a small amount of nutrients for cell growth. In order for the density of cultures to increase over time, they would need to be supported by the ingredients found in CBIS 1 Medium.

Experiment 2

Based on conclusions drawn from cultures in CBIS 1 Medium, a second series of experiments were conducted with CBIS 2 Medium. To address the potential absence of mineral and nutrient sources in Porewater 626-24, CBIS 2 Med was prepared by adding yeast extract to the medium following filter sterilization. Yeast extract should serve to replace nutrients lost during storage of the porewaters.

Resazurin was then added as an oxygen indicator and Na_2S was titrated into the CBIS 2 Med stock until the solution became anaerobic. CBIS 2 Med was then added to 30 mL anaerobic culture vials in the anaerobic hood. Culture vials were inoculated with the same culture isolates used with CBIS 1 Med, which represented isolates obtained from Het Med 1 at various depths throughout the core.

4.3.15 Culture Storage

Successful cultures were stored in 15% glycerol and DSMO. Glycerol stocks were prepared by adding 1.5 mL of liquid culture (or suspended plate culture) to 0.5 mL 60% glycerol. For DSMO storage, 1.8 mL culture was added to 0.2 mL 70% DSMO stock solution. Once prepared, glycerol and DSMO-stored cultures were placed in the freezer at -80°C.

4.3.16 Molecular Studies – Clone Libraries

An attempt to construct clone libraries of enrichment cultures was made in order to provide information about the diversity of enrichments and the potential types of species present in

cultures. DNA was successfully extracted from three samples originating from depths of 546.7 m (PM1 culture), 571.6 m (Het Med 1 culture) and 1608.8 m (Het Med 1 culture). DNA extractions were performed on enrichment cultures using the UltraClean™ Soil DNA Isolation Kit (MoBio Laboratories, Inc.) in the EGL Laboratories at the Open University, UK. DNA was amplified by PCR in a Perkin Elmer Geneamp 2400 thermal cycler with bacterial 16S rDNA primers pA/pH and pA/Com2 (Bruce *et al.* 1992; Brunk *et al.* 1996; Lane 1991; Schwieger and Tebbe 1998; See also **Chapter 5**). The PCR mix contained bacterial 16S rDNA primers, 5x Invitrogen Taq, 10X Buffer with Mg^{2+} dNTP (10mM) and Ultrapure sterile H_2O. Denaturing of DNA and enzyme activation was performed by heating samples to 94°C for 10 minutes. Thirty-five cycles for PCR were then performed:

1. Denaturing Step: 94°C at 1 min

2. Annealing Step: 55°C at 1 min

3. Extending Step: 72°C at 1 min (5 min for the final cycle)

Following PCR, samples were then run on an agarose gel and stained using ethidium bromide to confirm the presence of PCR product. Successful PCR amplification product was then gel purified.

Attempts to create clones using gel purified DNA were first made at the Open University, UK, using the commercially available TOPO® Cloning Kit (Invitrogen). When cloning attempts failed, a second round of PCR was run using pA/Com2 bacterial 16S rDNA primers to obtain a smaller fragment of DNA for cloning. Cloning attempts using this smaller fragment of DNA also failed.

Further attempts at obtaining clones using the commercial TOPO® Cloning Kit (Invitrogen) and TA Cloning® Kit (Invitrogen) were performed at the Voytek Microbiology Laboratory at the USGS in Reston, VA, USA. When initial attempts failed, internal fragments of the original, gel purified DNA amplification products were made using the primer sets shown in **Table 8**. Successful PCR product was obtained from only

the culture at depth 1608.8 m, dubbed CBIS 46E and obtained with the culture media Het

Med 1.

Table 8 - PCR amplification for clone libraries. Successful PCR products obtained with primers are indicated by "+". Unsuccessful attempts at PCR amplification are indicated by "-". "X" indicates that PCR was not attempted with the given primer at the depth listed.

Culture Labels	Depth (m)	46f / 1387r	46f / 519r	46f / 1100r	Geobacter	Sbr
			PCR Primer Sets			
CBIS 46E	1608.8	+	+	+	X	-
15 Iron Oxidizer Enrichment Culture	546.7	-	-	-	-	-
CBIS 16 E	571.6	-	-	-	X	-

4.4 RESULTS

The results of all tested culture medias across all CBIS cores used for inoculation are

shown in **Table 9**. Three of the media tested - IR DH media, SR Med 2 and SR USGS

media – showed no growth at any of the tested depths. All other media showed growth in

at least one replicate test for one depth. The most successful and consistent media for

obtaining cultures was Het Med 1, and the majority of enrichment cultures were obtained

using this media (**Table 9**).

Table 9 - Total number of depths from which successful cultures were obtained for each media type used.

Media Type	Total Cultures
Het Med DH	2
Het Med 1	12
Het Med 1 Plates	5
Aerobic Het Med 1 Plates	3
Humic Acid Utilisers	2
IR USGS	1
Iron Reducer DH	0
Iron Citrate Media	1
Sulfate Reducer DH	2
Sulfate Reducer Media 2	0
PM1 Medium	2
PM2 Medium	2
SR USGS	0
Fe Oxidizer Medium	1

4.4.1 Het Med DH

Het Med DH yielded cultures at two out of four depths in the post-impact sediments

resting above the impact structure (250.5 m, 366.6 m). Cultures in this media were difficult

to view due to particulate background and precipitates that were a natural product of the

media. All cultures appeared as clumps or mat-like collections containing numerous cells rather than single cells present in the media. When cultures were transferred to solid media on plates, or diluted into fresh liquid media, no additional growth occurred and cell cultures were lost. Cell cultures in the original liquid media were visible after 8 days, but then died off after 15 days. At the two successful culture depths, only 25% of replicate produced cultures at 250.5 m and 50% at 366.6 m. Across all replicates at all depths for Het Med DH, this represents a success rate of 25%.

4.4.2 Het Med 1

Out of all media tested, Het Med 1 yielded the largest number of enrichment cultures. Successful cultures were obtained from post-impact sediments at depths of 250.5 m and 366.6 m; in the upper resurge breccia and tsunami deposits at depths of 544.3 m, 571.1 m, 588.1 m, 614.6 m, 643.5 m and 842.1 m; and in the deepest section of the core below the granite megablock at depths of 1447.5 m, 1473.3 m, 1568.5 and 1608.8 m. In total, twelve out of twenty depths examined produced enrichment cultures.

Four of the cultures obtained with Het Med 1 - those from depths 571.1 m, 643.5 m, 1568.5 m and 1608.8 m - were consistently able to grow in replicate tests, and had success rates above 50% across all inoculations (see **Table 20** later in this chapter for success rates with all media types). Other cultures obtained with Het Med 1 were not as consistent, and their survival after being cultured was often low.

Five of the enrichment cultures obtained with Het Med 1 could be grown on solid media in plates. These include the four depths mentioned above with the addition of depth 842.1 m. However, even with samples from these depths, spreading growing cultures from liquid media onto solid plates was not always successful (**Table 20**). Only three of the five cultures had success rates above 50% on solid media. Only three of the cultures also showed growth in aerobic condition on plates, but with a significantly reduced success rate of 14% for all three (**Table 20**).

In aerobic tests, no inoculations of strictly aerobic liquid media showed growth over a period of one month (data not included in **Table 20**). This indicates that either no cultureable aerobic organisms were present in the CBIS sediments tested with liquid Heterotrophic Media 1, or that aerobic organisms did not grow freely in liquid media and were so closely associated with inoculate sediments that cells could not be distinguished from sediment particles. When anaerobic inoculations were prepared in liquid Het Med 1, 50 μL aliquots were also removed and spread on solid media plates. All depths that showed no growth of enrichment cultures in anaerobic Het Med 1 also showed no growth on aerobic Het Med 1 plates (**Table 20**).

Enrichment cultures that were successfully grown in anaerobic Heterotrophic Media 1 were also tested for their ability to survive in the ambient, oxygen-rich atmosphere on solid Het Med 1 plates (**Table 20**). A few of the enrichment cultures showed growth when spread on plates. This may indicate that aerobic, or facultative anaerobic organisms present in the sediments may be limited to growth on a substrate rather than in liquid media.

Organisms cultured in enrichments on aerobic plates were morphologically distinct from organisms present in the anaerobic enrichment cultures from which they were grown. This could mean that the aerobically grown organisms are inactive in anoxic conditions; or they are less competitive than the strictly anaerobic organisms grown in anaerobic cultures and therefore do not represent a large percentage of the anaerobic enrichment culture and are therefore not as prevalent in samples viewed under the microscope.

4.4.3 CBIS 16E

Enrichment cultures obtained from a depth of 571.1 m were dubbed CBIS 16E. The cells in these cultures appeared to be rod-shaped and possibly filamentous. Cells often clumped together in groups ranging anywhere from three cells to hundreds (**Figure 24** and **Figure 25** and **Figure 27**). Cells were not always associated with sediment particles, although it

was common to see sediments with numerous cells in such close proximity that they appeared to be attached (**Figure 26**).
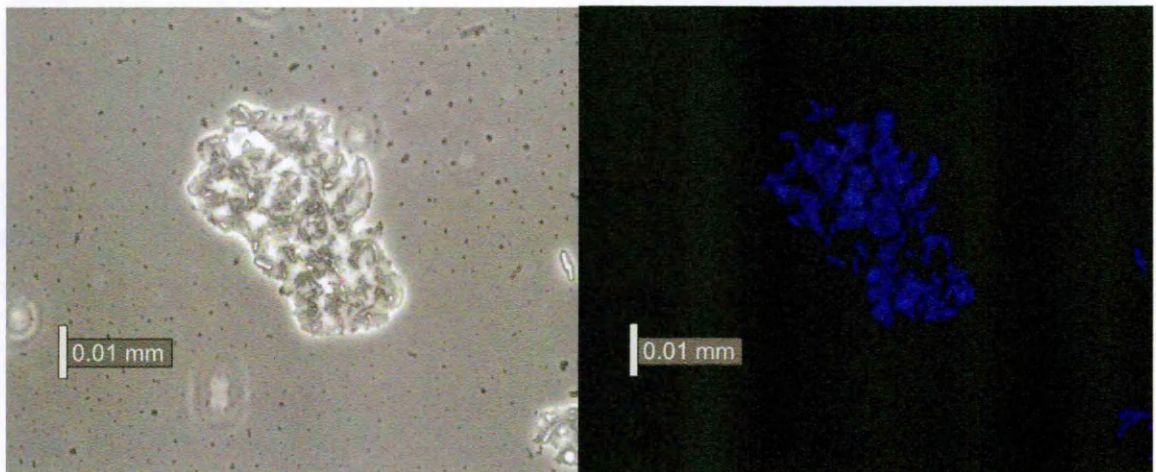
Figure 24 - Enrichment culture obtained from CBIS sediments at a depth of 571.1 m shown in white light (left) and stained with DAPI (right).
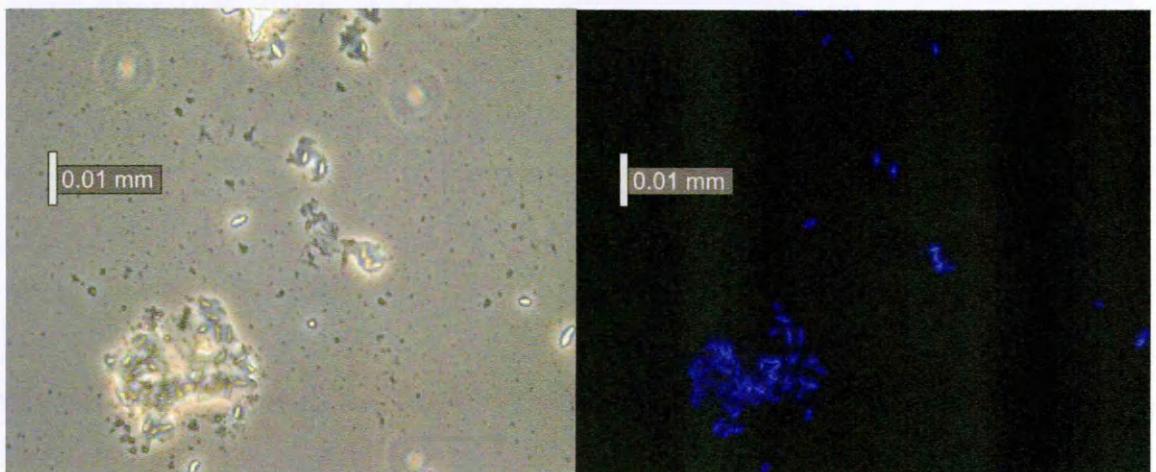
Figure 25 - Enrichment Culture obtained from CBIS sediments at a depth of 571.1 m. Single cells are visible away from the clumps.
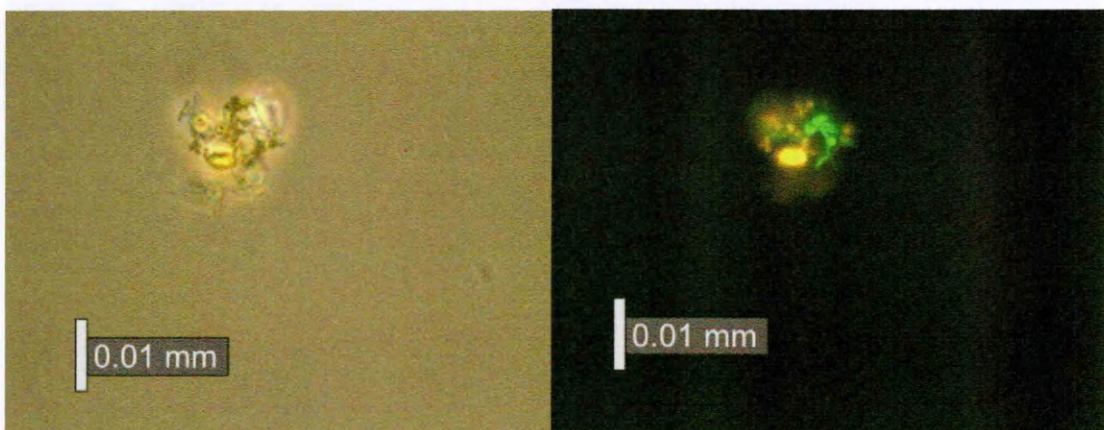
Figure 26 - Enrichment culture obtained from CBIS sediments at a depth of 571.1 m. Cells stained with Syto 9 glow green, and can be seen associated with CBIS sediment particles (yellow).
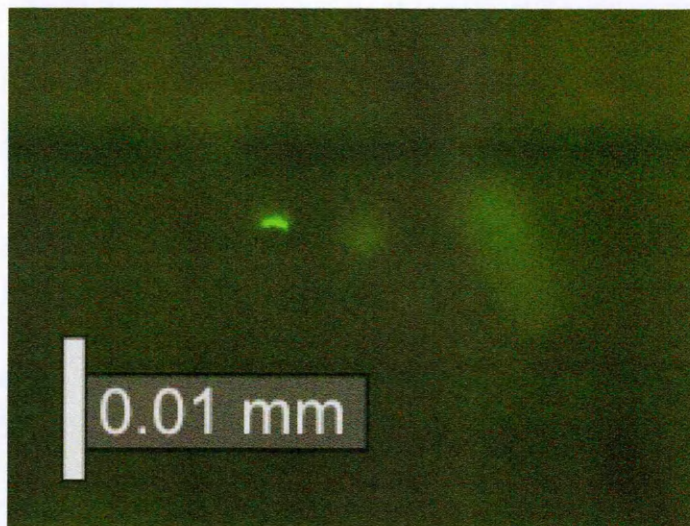
Figure 27 - An individual cell from CBIS 16E culture stained with the dye Syto 9.

4.4.4 CBIS 19E

Enrichment cultures obtained from sediments at a depth of 643.5 m were dubbed CBIS 19E. Unlike CBIS 16E, which originated from sediments at similar depth and with similar chemical and lithologic composition, cells in CBIS 19E did not tend to form large mats. Single cells could frequently be seen floating freely in solution (**Figure 28**). Cells could also be seen paired as apparently dividing cells, or in chains of three. It also appeared that two cell types were present. Small, coccoid cells could often be seen floating singly. Rod-shaped cells were more likely to be viewed as pairs. It could not be determined if these were distinctly different species, or different morphologies of one species type.
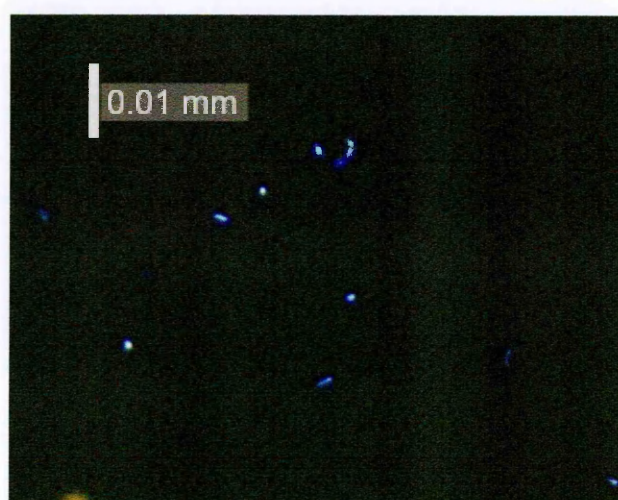
Figure 28 - Cells of enrichment cultures obtained from sediments at a depth of 643.5 m and stained with the fluorescent dye DAPI.

4.4.5 Humic Acid Utilisers

Humic Acid Utiliser media was inoculated onsite at the time of core sampling in two replicate culture bottles and at eight depths through the core. Successful cultures were identified by a colour change in the media (from light to dark). One successful culture was identified from a depth of 770.8, and both replicates appeared successful at a depth of 787.4. When the three culture samples were returned to the Open University for analysis, samples from cultures were viewed by epifluorescence microscopy using the DNA-binding dye DAPI in order to ensure that successful growth had occurred.

4.4.6 Iron Reducer Media Tests

As with the Humic Acid Utiliser media, two replicate bottles of Fe USGS media were inoculated at each selected depth of the CBIS core. Positive cultures were identified by a colour change in the iron precipitate present in the media (from light to dark) (**Figure 29**). Four depths showed positive growth with this method of identification. One replicate was positive at 677.8 m, two at 770.8 m, one at 821.0 m and two at 1447.5. When samples were returned to the Open University for analysis, only the two replicates from 1447.5 m appeared to have cells when viewed under epifluorescence microscopy, indicating that the other depths were false positives.

Figure 29 - USGS Fe Media showing colour change that indicates possible growth of cultures. Two bottles (right) contain darker iron precipitates, which was used as indication that these cultures warranted further examination.

CBIS core samples were used to inoculate a total of thirteen depths with IR DH media (see **Table 20**). No depths returned successful enrichment cultures.

Fe Citrate Med was inoculated with samples of core sediments from depths of 250.5 m and 366.6 m with iron citrate concentrations of 76 mmol and 17.6 mmol. Successful cultures were difficult to determine in this media, due to the formation of particulate precipitates that often appeared cellular in shape and interfered with DAPI and Syto-9 fluorescence when attempts were made to stain cultures. However, it appears that one successful enrichment culture was obtained at a depth of 366.6 m and a concentration of 17.6 mmol iron citrate. In total, this means that only 25% of all replicate tests with Fe Citrate Med yielded cultures.

4.4.7 Sulfate Reducer Media Tests

Attempts to identify successful cultures in sulfate reducing media were performed in two ways. First, cells were identified by viewing cells under epifluorescence microscopy. Secondly, a strip of lead acetate paper was used to identify the presence of sulfide, which is produced by sulfate-reducing bacteria, in cultures according to a procedure by Bekins *et al.* (1999). Briefly, a 1 mL aliquot of media was removed from culture bottles inside the anaerobic hood and placed in a pre-autoclaved sterile Eppendorf tube. 50 μL of 1 M NaOH was then added to the aliquot to convert hydrogen sulfide (HS^-) into sulfide (S^{2-}). Lead acetate paper was then dipped into the aliquot. Darkening of the paper (from white to grey/black) indicated the present of sulfide.

With the first attempt at culturing with SR DH media, the 0.2M Na_2S was removed in order to reduce the amount of precipitate forming naturally in the medium. Additionally, Na_2S would provide a source of sulfide in the media, and could potentially cause a false positive result with the lead acetate method of culture detection, which would react to the sulfide even though it was not biologically produced. Sulfate reducers produce sulfide via metabolism, which can appear as a black precipitate in the medium. Addition of sulfide in the form of 0.2M Na_2S to reduce oxygen in the medium also causes the formation of a

black precipitate, which makes identifying cells more difficult. Without the addition of

Na2S, the formation of precipitate could also be used as an indicator of microbial

metabolism.

Cultures were examined with lead acetate paper following 44 days of incubation in

39°C. All cultures appeared negative. When viewed under 10X magnification, cultures

from a depth of 571.1 m were the only culture that appeared to have organisms present

(**Table 10**). However, the cells appeared similar to those seen in Het Med 1. After re-

examining the media, it was decided that the presence of sodium lactate may provide a

source of energy for organisms that are able to ferment lactose and not exclusively

sulphate reducing microbes. Lactose fermenting microbes have been found to be present in

a number of environments, including seawater (Oliver 1983). It is possible that similar

organisms could be present in the porewaters of the CBIS. For this reason, it was

determined that a second attempt at culturing organisms in SRM 1 should be made after

removing sodium lactate from the recipe.

Table 10 - First round of culturing using SR DH media. Two replicates were prepared for
each depth. Four replicate controls were prepared.

Depth (m)	Lead acetate	Microscope
571.06	-	+
643.48	-	-
997.73	-	-
1473.26	-	-
1568.54	-	-
1608.76	-	-
1608.76	-	-
1608.76	-	-
1608.76	-	-
Controls	-	-

A second batch of cultures with no lactose was prepared using samples from

depths: 2, 5, 8, 12, 16, 17, 18, 19, 20, 33, 36, 45 and 46. Sediment from these depths was

placed in 30 mL anaerobic culture bottles. Up to 15 uL of 0.2M Na2S was added to some

bottles to reduce any oxygen present (as indicated by resazurin), and did not produce any

noticeable amount of precipitate. Any bottles to which 0.2M Na2S was added were

marked. A final check for growth was performed after 238 days of incubation at 39°C.

Table 11 - Second round of culturing using SR DH media with lactose removed. Two replicates were prepared for each depth. Depths marked (*) are those where Na₂S was added. Results marked "?" indicate that a positive result could not be given definitively by the method used to detect cultures.

Depth (m)	Lead acetate	Microscope
159.26	-	-
250.48	-	-
*366.61	+	?
*449.29	-	-
544.27	-	-
571.06	-	-
588.08	-	-
614.56	-	-
*677.77	-	-
*997.73	-	-
1085.45	-	-
1568.54	-	-
1608.76	-	-
Controls	-	-

As with other media adapted from D'Hondt (2003) for seafloor sediments, SR DH media had very limited success in culturing organisms from CBIS cores. Only two out of seventeen depths tested had cells present after incubation (366.6 m and 571.1 m). For these two depths, only 17% and 25% respectively of replicates showed successful cultivation. Depth 366.6 m was not given a positive for culture growth in the final results (**Table 20**) because only the lead acetate method of detection showed any indication of microorganisms being present. The lead acetate method likely provided a false positive because Na₂S was added to the culture media at this depth and would have produced small amounts of sulfide.

SR USGS media showed no growth in the eight depths that were tested. Similarly, of the five depths tested with SR 2 media, none showed any sign of successful cultures after incubation (**Table 20**).

SR 2 Med was inoculated after data on the lithologies and chemical composition of core porewaters were obtained from the USGS. The range of depths tested with SR 2 Med were selected because they showed a high level of sulfide in chemical data provided by researchers at the USGS in Reston, VA. It was thought that elevated levels of sulfide could be a product of microbial respiration.

As with SR 2 Med, PM1 and PM2 media were tested at depths where chemical data showed a high concentration of sulfide in CBIS cores. Of the five depths tested, 544.3 m showed growth with PM1 Med, 588.1 showed growth with both PM1 and PM2, and 643.5 m showed growth with PM2. The highest success rate (67%) was with PM1 at depth 544.3 m. This depth represents the highest success rate in culture replicates for sulfate reducing microorganisms.

4.4.8 Methanogenic Media Tests

Both media types for methanogens, H2 Methano and A Methano, showed no culture growth for all depths tested. For the Acetate Methano Media for methanogens, two depths (900.9 m and 1447.4 m) showed a slight signal for methane production but it was below the detectable limit for the analysis method used.

4.4.9 Iron Oxidizer Media Tests

Depths for IO Media were selected based on the high content of iron observed in chemical data provided by researchers at the USGS in Reston, VA. One successful culture was obtained using IO Media at a depth of 1419.1 m. Further depths did not show any cell growth after incubation. This culture showed a success rate of 67% across the attempted replicate inoculations.

4.4.10 Impoverished zone Tests

When sediments from the biologically 'impoverished zone' between depths of 867 meters to 1096 were inoculated with successful enrichment and isolate cultures obtained at other depths in the CBIS, successful growth was observed in both cases (**Table 9**). For liquid impoverished zone tests, after 150 days of cultivation at 37°C, three of the inoculations showed growth in both replicates, one inoculation showed growth in only one of the two replicates, and all controls showed no growth (**Table 12**).

Table 12 - Liquid Media containing sediments from 997.7 m as a source of carbon inoculated with CBIS microbial cultures. (+) indicates successful growth after 150 days of incubation. (-) indicates no growth.

Culture Type	Depth (m)	Results	Cells/mL (150 days)
Controls	Controls	-	0
Isolate	571.1	+	5.8E+03
Isolate	571.1	+	1.4E+04
Isolate	643.5	+	2.9E+04
Isolate	643.5	-	0.0E+00
Enrichment	842.1	-	0
Enrichment	842.1	-	0
Enrichment	1568.5	+	7.7E+03
Enrichment	1568.5	+	7.7E+03
Isolate	1608.8	+	4.4E+04
Isolate	1608.8	+	3.8E+03
Enrichment	1608.8	-	0
Enrichment	1608.8	-	0

Successful cultures were then spread on plates containing solid Het Med 1 (four replicates each) in an attempt to re-grow colonies on solid media. This proved unsuccessful in all replicates.

Inoculation 1 of slurry cultures proved similarly successful, showing growth from all but two inoculations (**Table 13**). Slurries were successful when inoculated with both

Table 13 Slurry cultures of Inoculation 1 using sediment from a depth of 997.7 m as a nutrient source

Culture Inoculate	Depth from which culture was obtained	Successful Growth (+/-)	Liquid Sample Preparation
Control	N/A	-	Sterile H_2O
46 Enrichment	~1609 m	+	200 µL of liquid culture used
46 Isolate	~1609 m	+	Plate culture suspended in 1 mL sterile Het Med 1
45E (1)	~1568 m	+	Plate culture suspended in 1 mL sterile H_2O
45E (2)	~1568 m	+	Plate culture suspended in 1 mL sterile H_2O
45 Cross Inoculation	~1568 m	-	Plate culture suspended in 1 mL sterile H_2O
19 Enrichment	~643.5 m	+	200 µL of liquid culture used
19 Isolate	~643.5 m	+	Plate culture suspended in 1 mL sterile H_2O
16 Enrichment	~571 m	+	200 µL of liquid culture used
16 Isolate	~571 m	-	Plate culture suspended in 1 mL sterile H_2O

liquid cultures and with plate cultures re-suspended in liquid Het Med 1. **Figure 30** shows

cells from an enrichment culture at 1568.5 m growing in sediments from 997.7 m. Cells

were stained with DAPI following the procedure for DAPI staining of enumeration

samples (see **Chapter 3**).
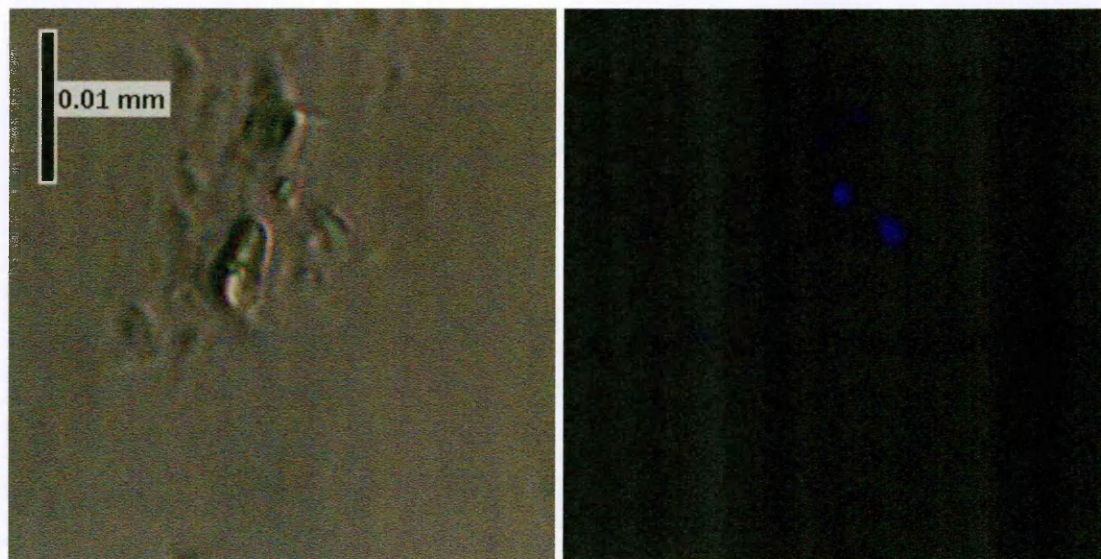
Figure 30: Heterotrophic enrichment culture obtained from a depth of 1568.5 meters growing in a slurry culture (Inoculation 1) using core material from a depth of 997.7 meters. Images are shown in white light (left) and stained with DAPI (right).

In **Inoculation 2** of slurry cultures, no indication of growth was observed (**Table 14**).

Table 14 - Slurry cultures of Inoculation 2 using sediment from a depth of 997.7 m as a nutrient source

Culture Type	Depth from which culture was obtained	Successful Growth (+/-)	Notes
Control	N/A	-	None
Enrichment	571.1 m	-	50 µL of liquid culture added to slurry
Enrichment	842.1 m	-	50 µL of liquid culture added to slurry
Enrichment	1568.5 m	-	50 µL of liquid culture added to slurry
Isolate	1608.8 m	-	Plate culture was added to 50 µL sterile H_2O and added to slurry

4.4.11 CBIS Medium

Results for Experiment 1

All attempts to culture organisms in CBIS 1 Medium were unsuccessful (**Table 15**). This

may be due to the fact that high amounts of Na_2S were required to obtain anaerobic

conditions in the culture vials. Each 30 mL vial contained on average 2 mL of 0.2 M Na2S.

This level of Na_2S could prove toxic for cultures. Additionally, although the cultures

chosen for inoculations were those that proved easiest to culture in the laboratory, none

were from depths similar to Porewater 626-24. It is possible that Porewater 626-24 does

not provide the necessary environment for the selected cultures to survive. A final

consideration is that nutrient sources in Porewater 626-24 may have been entirely depleted.

If organisms were present in Porewater 626-24 during storage and transport from the

United States to the Open University, nutrients may have been used up prior to filter

sterilization. Further experiments were conducted to address some of these issues.

Table 15 Results of Experiment 1 using CBIS 1 Medium

Inoculation Culture	Depth (m)	Replicates	Result
16	571	2	-
19	643.5	2	-
45	1568.5	2	-
46	1608.7	2	-
Neg Control	N/A	2	-

For CBIS Med 2, where yeast extract was added to replenish lost nutrients in porewater,

cultures originally from depths of 643.5 m and 1568.5 showed successful growth (**Table**

16).

Table 16 - Results of Experiment 2 using CBIS 1 Medium

Inoculation Culture	Depth (m)	Replicates	Result
16	571	2	-
19	643.5	2	+
45	1568.5	2	+
46	1608.7	2	-
Neg Control	N/A	2	-

4.4.12 Clone Libraries

TA cloning successfully produced clones using fragments obtained with primers 46f/519r.

In total, 20 clones were obtained. DNA was extracted from these clones, amplified and

sequenced. Unreadable sequences were discarded, leaving 14 useable sequences that were

used to construct a phylogenetic tree rooted with a known sequence from the

alphaproteobacterium *Sinorhizobium* (**Figure 31**).
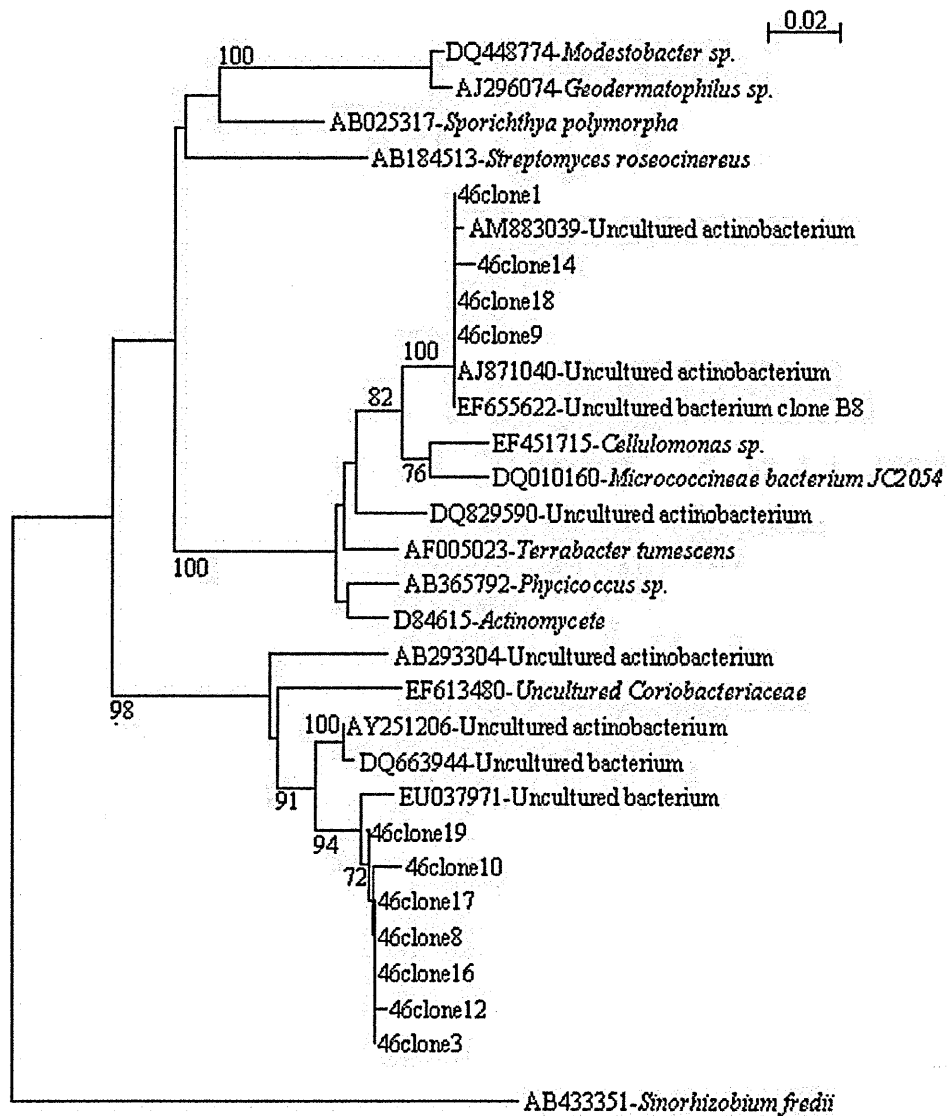
Figure 31- Phylogenetic tree constructed from clones derived from the enrichment culture CBIS 46E. The tree is rooted using the alphaproteobacterium *Sinorhizobium* with bootstrap values above 70 labelled.

Based on the construction of the phylogenetic tree, clones could be divided into

two general groups. Clones 1, 9, 14 and 18 appeared to be most closely related to

Cellulomonas and *Micrococcineaea bacterium* based on closest matching sequences from

the NCBI nucleotide Blast database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (**Figure 31,**

Table 17). Clones 3, 8, 10, 12, 17 and 19 were found to match up with a selection of

uncultured bacterial clones (**Figure 31, Table 17**). Many of the closest related species

from clones in Group 1 correspond to studies performed on soil, subsurface sediments or

marine sediments (**Table 17**). Sequences corresponding to Group 2 are also derived from

soil environments, and are often associated with degradation of petroleum products (**Table**

17).

Table 17 - Closest matching sequences for clones of CBIS 46E. Only the closest matching sequences from identified sources are shown.

Clones (CBIS 46E)	Closest Related Sequence (Accession number for the NCBI database)	Source of Related Sequence	Reference for Related Sequence
Group 1: (46clone1, 46clone9, 46clone14, 46clone18)	AM883039 – Uncultured actinobacterium	Prokaryotic community in the deep terrestrial sedimentary biosphere supported by coal layers.	Unpublished
	EF451715 – *Cellulomonas sp.*	Bacterial community in high Arctic permafrost soil from Spitsbergen, Norway	Hansen *et al.* 2007
	DQ010160 – *Micrococcineae bacterium JC2054*	Tidal flat sediments, South Korea	Yi *et al.* 2007
	AF005023 – *Terrabacter tumescens*	*Terrabacter tumescens*	Yoon *et al.* 2007
	AB365792 – *Phycicoccus sp.*	Ginseng rhizosphere soil in Korea	Unpublished
	AB025317 – *Sporichthya polymorpha*	Soil bacteria isolate	Tamura *et al.* 1999
	AJ296074 – *Geodermatophilus sp.*	Stones and monuments in the Mediterranean basin	Urzì *et al.* 2001
	DQ448774 – *Modestobacter sp.*	Marine sediments	Gontang *et al.* 2007
Group 2: (46clone3, 46clone8, 46clone10, 46clone12, 46clone16, 46clone17, 46clone19)	EF613480 – *Uncultured Coriobacteriaceae*	Benzene mineralizing bacterial communities under sulfate-reducing conditions	Kleinsteuber *et al.* 2008
	AB293304 – Uncultured actinobacterium	Acetate-assimilating microorganisms in anoxic rice field soil	Unpublished
	AY251206 – Uncultured bacterium	Diesel fuel-contaminated laboratory microcosms	Unpublished
	DQ663944 – Uncultured bacterium	Petroleum-contaminated sediments	Allen *et al.* 2007
	EU037971 – Uncultured bacterium	Anaerobic microbial conversion of residual oil to natural gas using an oil-degrading methanogenic bacterial consortium from a marginal sandstone reservoir core	Gieg *et al.* 2008

4.5 DISSCUSSION

On the whole, cultures that were obtained from regions in the CBIS core matched the Microbiological Zones defined by direct cell enumeration estimates (see **Chapter 3**). This lends weight to the idea that microbial re-colonization occurred after the sterilizing thermal heat generated by the impact dissipated. It also confirms the idea that Microbiological Zone 2 could be a biologically impoverished zone where there is no indication of indigenous microorganisms based on enumeration results, and where no culturing efforts in this study produced viable cultures. It is possible that areas in this region of the core

were sterilized by impact, and that re-colonization by microorganisms has been limited and microbial biomass in this region is low enough to avoid detection.

The easiest organisms to culture in CBIS cores were obtained using general heterotrophic media, specifically the Heterotrophic Medium 1 (Het Med 1) adapted from Stevens *et al.* (1995) and Tsai *et al.* (1995). This is expected, as general heterotrophs are able to utilize a wide range of electron donors and their requirements for growth are oftentimes less specific than those that require specific redox pairs, such as Fe(II)/Fe(III), and chemolithoautotrophs.

Heterotrophic organisms require a source of organic carbon, meaning that supplies of organic carbon have not been entirely depleted in the deepest section of the core where cultures were still present. According to the data concerning DOC content in core porewater provided by the USGS, dissolved organic carbon is also detectable at these depths. Typically, supplies of organic carbon are expected to be reduced with depth and age of sediments as, over time, they are degraded by heterotrophic microorganisms. Organic carbon could be provided by influx of groundwater, although according to hydrological data obtained by the USGS, movement of water at these depths is still low (Sanford *et al.*, *In Press*). It is possible that the action of impact cratering, and subsequent mixing of materials from the subsurface and the tsunami resurge, acted to 'recharge' supplies of organic carbon at depths that would otherwise be depleted. Here, further data on DOC at depth in undisturbed Atlantic Coastal Plain sediments would be of great interest as a comparison.

Cultures obtained with the Humic Acid Utiliser media (HA Med) were concentrated around depths near the bottom of Microbiological Zone 1 with one culture at a depth of 770.7 m and two at 787.3 m. This is a region composed of sediment megablocks with diamicton intercalations, and is interpreted as an area of material deposited by the tsunami resurge with a mix of sediment megablocks that collapsed into the crater from the rim. Data provided by researchers at the USGS in Reston, VA, concerning dissolved

organic carbon (DOC) throughout the core shows relatively high levels of DOC at most depths (Sanford *et al.*, *In Press*). DOC includes components of marine humics, humics, fulvic acids, tyrosine and tryptophan. Interestingly, there is a spike in total DOC at a depth of 784.6 m that corresponds to the depths of successful cultures (**Figure 32**). According to the results obtained by the USGS main components of total DOC at this depth are fulvic acids at 33%, which could provide a source of nutrients and carbon for microorganisms (Sanford *et al.*, *In Press*). This supports the idea that cultures obtained with HA Med are indeed utilizing humic substances for growth. However, humic substances compose a large portion of DOC at all depths, and levels are high enough throughout the core for growth. Measurements of dissolved organic carbon in porewaters are not necessarily an indication that organic carbon is available for microbial respiration. In a study of cores from Atlantic Coastal plain sediments in South Carolina, no correlation between microbial-produced CO_2 and total organic carbon content in the sediment was found (McMahon et al. 2005). DOC levels alone do not provide an explanation for why HA Med only yielded cultures at depths limited to the bottom of Microbiological Zone 1.
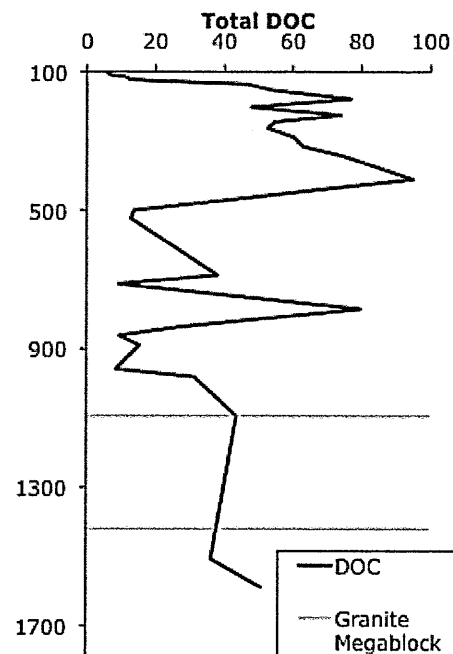
Figure 32 - Total DOC in porewaters from CBIS core samples. Data provided by the Voytek Microbiology Lab and collaborators at the USGS.

4.5.1 Iron

Three media for iron reducers were used, two of which yielded cultures at only one depth respectively. IR Citrate media provided cultures in only 50% of replicates at 366.6 m. Cell densities in these cultures were very low, even after significant incubation times. When these cultures were transferred to fresh media, no growth occurred. It's difficult to say if these cultures are truly iron reducers, and could feasibly be general heterotrophs growing in the IR Citrate media.

IR USGS media obtained cultures at a single depth of 1447.5 m in 100% of replicates. Unlike the organisms in IR Citrate Med, the IR USGS cultures grew in dense numbers and could be cross-inoculated into fresh media to perform dilutions. This enrichment culture was also successfully grown on solid IR USGS media. Again, although IR USGS media is designed for growth of iron reducers, it does not ensure that these organisms are strictly iron reducing.

Iron content in porewaters throughout the CBIS core according to data obtained from the USGS in Reston, VA, is shown in **Figure 33**. Previously, many studies have cited Fe(II) accumulation in subsurface environments as an indicator of biological reduction of Fe(III) (e.g. Jones et $al.$ 1983; Lovley and Phillips 1986; Lovley et $al.$ 1990; Nealson and Saffarini 1994; Zhang et $al.$ 2005). At a depth of 366.6 m, Fe (III) levels are low at 0.06 μmol /g while Fe (II) is 1.7 μmol. This may indicate the reduction of Fe(III) to the less environmentally stable Fe(II), which would be consistent with the idea that IR Citrate Med was successful in culturing iron reducers. This region of the core is composed of Eocene marine deposits, and is less likely to be strictly anaerobic than deeper areas of the core where hydrologic conductivity is low. Additionally, the pH is a relatively neutral 7.3. Fe (II) is naturally converted to Fe (III) in aerobic and in neutral environments. This suggests that Fe(II) content may be higher than Fe(III) because there is some form of active conversion maintaining Fe(II) levels, such as microbial metabolism.

Just below this depth, at 374.3 m, Fe(III) rises steeply to 3.63 μmol/g. The chemical conditions and pH of sediment do not change drastically between these depths. This could indicate that reduced iron is being oxidized back into Fe(III) as the elements seep downward. Unfortunately, culture samples were not collected from a depth of 374.3 m and media for iron oxidizers (Fe Oxid Med) could therefore not be tested at this depth. The next closest depth from which samples were collected is at 435.6 m where Fe(II) peaks once again. If iron reducers are indeed present at 366.6 m, it is possible that iron oxidizers have established themselves just below to take advantage of an input of Fe(II) for oxidation.

The other successful culture for iron reducers, obtained with IR USGS Med, is from a depth of 1447.5 m. At this depth, Fe (II) levels are raised once again to a level of 3.8 μmol/g (**Figure 33**). Fe(II) is generally raised in this region of the core, which corresponds to Microbiological Zone 3 and the suevite, schist and pegmatite at the base of the CBIS core. Because IR USGS Med was inoculated at the time of core collection, samples at varying depths were somewhat randomly selected due to the availability of materials on site. This depth was the only sample inoculated from this region of the core, although chemical data suggests that if iron reducers are able to grow at this depth, they would likely be successful at other depths in the region as well.

Attempts to culture iron oxidizers, which oxidize Fe(II) to Fe(III), were performed after chemical data on the core became available. Inoculations of Fe Oxid Med were selected from the bottom region of the core, where iron levels were the highest. A closer look at this region, from 1419.1 m to 1677.3 m, reveals dramatic shifts in the elevated Fe (II) levels with depth (**Figure 33**). This fluctuation in Fe(II) occurs in spite of the fact that levels in pH remain neutral, and indicates that redox changes may be occurring as a result of microbial action. One successful culture was obtained from 1419.1 m, at the very top of this region of the core. Further depths were tested at high and low points of Fe(II), none of which yielded successful cultures.

Iron Content of CBIS Cores
umol/g
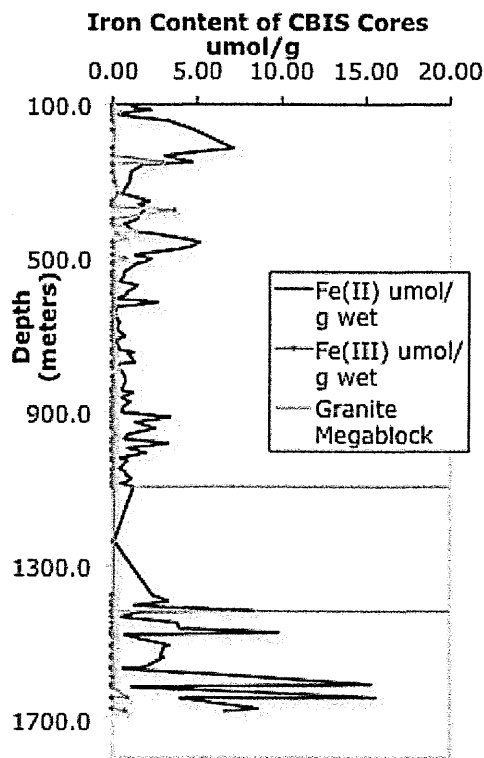
Figure 33 - Iron content in porewaters extracted from CBIS cores. Orange indicates Fe(III) while brown indicates Fe(II). Data provided by Ward Sanford and collaborators at the USGS.

Again, there is no guarantee that cultures obtained from iron reducer and iron oxidizer media are strictly limited to these forms of metabolism. It is possible that general heterotrophs could successfully grow in the media, particularly if sediments used to inoculate the media carried with them necessary nutrient and carbon inputs. Further tests on cultures were performed to identify species within the enrichment cultures with higher confidence (see **Chapter 5**).

4.5.2 Sulfate Reducers

Four different media were used to culture potential sulfate reducers from depths throughout the CBIS core. In general, studies on core porewaters show that the upper section of the core has high levels of sulfate relative to the rest of the core according to chemical data from the USGS in Reston, VA (**Figure 34**). A dramatic decline in sulfate occurs below 459.3 m followed by a sharp increase at 583.1 m and decrease at 594.7 m. Below 700.7 m, sulfate fluctuates within a more limited range to the bottom of the core.

The first media tested was SR USGS, which was inoculated on site at the time of core collection. Depths used for SR USGS media were below 677.8 m and no inoculations yielded cultures. By chance, these inoculations were all from depths where sulfate was quite stable, roughly between 2 and 3 mg/L. The most likely depths in which to find sulfate reducers are those where sulfate levels drop suddenly. It is therefore not surprising that SR USGS media did not yield any cultures from the depths selected for inoculations.

SR DH media was tested at many more depths. Data on sulfate concentration in the core was not available when SR DH media was tested, so inoculations were made throughout the length of the core. Only two depths showed any indication of cultures, both with very low replication. Only 17% of replicates at 366.6 m and 25% of replicates at 571.1 m showed any indication of growth. Obtained cultures were also difficult to cross inoculate into fresh media and had low survival rates after the first indication of growth, and were therefore difficult to characterize any further. Because cell densities were so low and the cultures were very difficult to replicate, confidence was low that cultures obtained with SR USGS were in fact legitimate.

SR Med 2, PM1 and PM2 media were all inoculated after data on sulfate concentration in cores was available from the USGS. Based on this data, the most interesting depths in terms of sulfate appeared to be in the region of decline and increase between 459.3 m and 700.7 m. Depths of 544.3 m, 571.1 m, 588.1 m, 643.5 m and 677.8 m were selected for inoculations. SR Med 2 was unsuccessful at all depths tested. SR Med 2 was the most complicated media used in tests, requiring eight carefully prepared solutions that included some highly toxic trace elements (**Appendix II**). Preparation of SR Med was a sometimes difficult and delicate procedure as solutions were produced and mixed. The complications involved with preparing the media may have resulted in negative results, as presumably any imbalance in components might yield a final solution that is toxic to microorganisms.

PM1 and PM2 were moderately successful in obtaining cultures from the selected depths. Cultures were obtained from depths of 544.3 m and 588.1 m with success in replicates of 67% and 50% respectively. 544.3 m rests at the very bottom of the largest decline in sulfate (**Figure 34**), which was the depth most expected to yield sulfate reducers. 588.1 m rests where sulfate level begin to peak again after the decline around 544.3 m. PM2 media showed cultures at 588.1 m 643.5 m with success of 50% and 33% respectively. 643.5 m is found at another low in sulfate.

Again, obtaining cultures in PM1 and PM2 media does not guarantee that they are indeed sulfate reducing bacteria. However, because these cultures were found around the largest shift in sulfate levels it would seem possible that their growth is somehow related to the processing of sulfate. PM1 544.3 sits at the point of lowest sulfate in the core, when levels drop from 5.18 to 0.8 mg/L. Cultures at this depth were also replicable with a relatively high level of confidence, allowing for further studies to try and characterize the species present in enrichments (see **Chapter 5**).
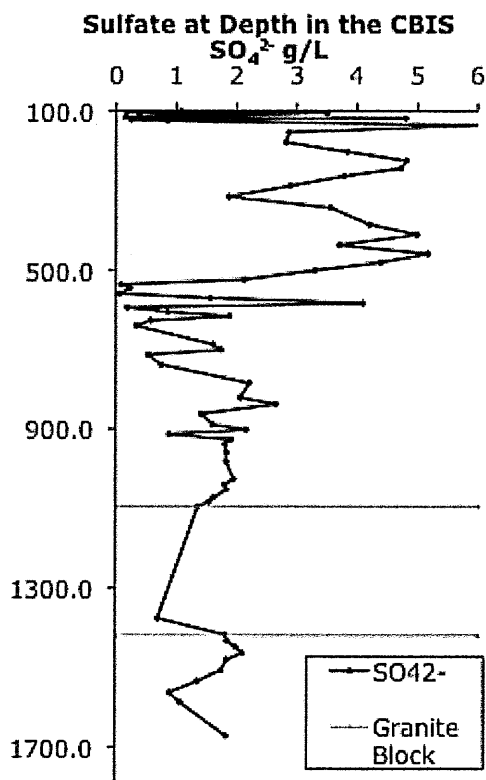
Figure 34 - Sulfate as measured in porewater drawn from CBIS cores. Data provided by Ward Sanford and collaborators at the USGS.

4.5.3 Onsite Inoculations

The selection of media that were prepared prior to core operations, and which were inoculated onsite at the time of core collection are listed in **Table 18** alongside the results. The majority of these media returned no cultures, including media for sulfate reducers (discussed above) and methanogens.

Acetate Methano Media had a slight signal at depths of 900.9 m and 1447.4 m, but it was well below the detection limit required for a positive results and was attributed to background. Methanogens were expected to be present in regions with a high methane (CH_4) signal. Based on data derived from core porewaters, a large peak in methane occurs at 366.4 m. Unfortunately, as methane concentration data was not available at the time of core collection, this depth was not tested with methanogen media. This is the only significant methane peak observed in data on the CBIS core, and it is possible that methanogens are not present in this environment or that the depths that they inhabit occur in un-sampled areas between the cores collected for biological studies.

Table 18 - Results of pre-prepared media inoculated onsite with sediment from CBIS cores. (-) represents no indication of growth. (+) represents successful growth. (X) indicates that tests for successful growth were not strong enough to confirm that cultures had successfully grown. N/A indicates samples that were not inoculated. Highlighted samples are those where cells were easily identified using epifluorescence microscopy. Samples marked 'N/A' indicate that replicates were not successfully inoculated.

Depth	Sample Name	Humic Acid Utilisers	SR USGS	IR USGS	H2 Methano Media	Acetate Methano Media
677.7	20-1	-	-	X	-	-
	20-2	-	-	-	-	-
702.5	21-1	-	-	-	-	-
	21-2	-	-	-	-	-
744.5	22-1	-	-	-	-	-
	22-2	-	-	-	-	-
770.7	23-1	-	-	X	-	-
	23-2	+	-	X	-	-
787.3	24-1	+	-	-	-	-
	24-2	+	-	-	-	-
821.0	25-1	-	-	-	-	-
	25-2	-	-	X	-	-
900.9	30-1	-	-	-	-	-
	30-2	-	-	-	-	X
1447.4	39-1	-	-	+	-	X
	39-2	N/A	-	+	N/A	X

Inoculations performed at the time of core collection were to take advantage of materials available in the onsite microbiology laboratory. Inoculations were blind in that no data concerning core materials, chemistry and levels of contamination were available at the time of collection. These inoculations are useful in that sediments had undergone the least amount of manipulation in terms of preparation, storage and transport.

Successful cultures in humic acid utiliser media and iron reducer media appear to match depths where such metabolic process would be possible according to chemical data obtained from core porewater samples. It is possible that these media were successful in culturing their target microbial communities.
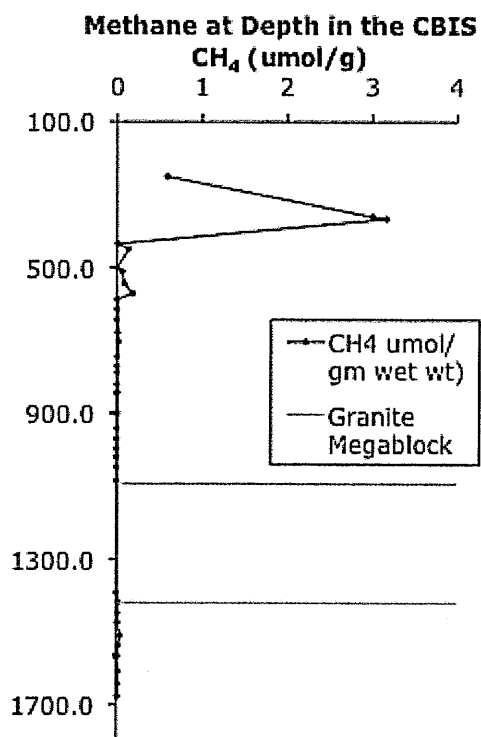
Figure 35 - Methane in the CBIS derived from analysis of core porewaters. Data provided by Ward Sanford and collaborators at the USGS.

4.5.4 Impoverished zone Tests

Direct counts of cells in CBIS cores using DAPI staining reveals little to no cells at depths between depths 867 meters to 1096. This region of the core is defined as Microbiological Zone 2 (**Chapter 3**), and is considered to be an 'impoverished zone' where enumerations, culturing and molecular studies have revealed no definitive indication of indigenous

microorganisms. However, DOC analysis indicates that carbon should be present at these depths to provide for microbial growth (see chemical data provided by the USGS in **Figure 32** and **Figure 37**). Therefore, one would expect to find some indication of microbial populations present in these samples, especially when considering that microbes are present at depths above and below this region.

Tests were performed to determine whether or not sediments from the impoverished zone are capable of supporting microbial growth. To do this, these sediments were inoculated with successful cell cultures obtained from other depths in the CBIS core. Both enrichment cultures and single species isolates obtained with Het Med 1 were used for inoculations in both liquid and slurry media. Liquid media was made by removing carbon sources from Het Med 1 and replacing them with autoclaved sediment from a depth within the Impoverished zone of 997.7 m. Slurry media was made by adding a small liquid inoculate, either from liquid cultures or by re-suspending plate cultures in liquid, to dry sediments from a depth of 997.7 m. Both un-autoclaved and autoclaved sediments from 997.7 m were used to make slurries.

Cultures were successfully grown in both the liquid and slurry Impoverished zone tests (**Table 19**), indicating that sediments from within the Impoverished zone is capable of supporting microbial growth and that sediments from this depth are not toxic to microbes native to the CBIS. Un-autoclaved sediments in slurry cultures supported growth, but it is possible that the addition of small amounts of media, or dead cells themselves in inoculate cultures, provided nutrients required for growth of indigenous organisms from depth 997.7 m. This is not likely, as all culturing attempts from 997.7 m using Het Med 1 were unsuccessful. To make sure that sediments from 997.7 m were sterile, sediments were autoclaved to make a second batch of slurry cultures. When autoclaved sediment from 997.7 m was used to make slurries, no apparent cells could be viewed under the microscope indicating that these slurries were unsuccessful. This implies that necessary nutrients contained in sediments from 997.7 m were destroyed when sediments were

autoclaved. Only a small amount of nutrient sources in media and dead cells would have been added to the dry sediments to make the autoclaved slurries, and would not have been sufficient to replace lost nutrients in 997.7 m sediments during autoclaving. Although carbon sources were still present in sediments, this lack of nutrients prevented growth.

Autoclaved sediments were also used to make liquid media for inoculations, and these tests showed successful growth of cells. These liquid cultures were made using Het Med 1 with carbon sources removed. However, unlike the autoclaved slurry cultures, the media still contained significant vitamins and trace minerals, providing sufficient amounts to replace the nutrients from 997.7 m lost through autoclaving.

Although cells were visible in many of the liquid Impoverished zone tests, only minimal growth had occurred. After 150 days of incubation, cell numbers were still quite low.

When successful liquid inoculates in this test were re-plated on solid media, none of them grew. This was not surprising, as all cultures used to inoculate the liquid Impoverished zone tests were originally from liquid cultures themselves. Additionally, growth in the Impoverished zone tests was low, indicating that cell cultures were not particularly robust and it is likely that the stress of transfer to plates and the low cell density of media spread on plates prevented successful growth on the solid media.

Table 19 - Results of culturing experiments using porewaters drawn from CBIS cores and sediments from the 'Impoverished zone' of Microbiological Zone 2. Depths marked "-" indicate inoculations that did not show growth. Shaded depths are inoculations that showed successful growth. Numbers indicate the percentage of replicate inoculations that were successful. Hatched boxes indicate depths where inoculate cultures were not used for the selected media.

Depth from which inoculate cultures originated (Meters)	CBIS 1 Medium	CBIS 2 Medium	Impoverished zone Slurries	Impoverished zone Liquid Tests	Impoverished zone Liquid Tests on Plates
571.1	-	-	50	67	-
643.5	-	100	100	67	-
842.1	(hatched)	(hatched)	(hatched)	67	-
1568.5	-	100	67	50	-
1608.8	-	-	100	50	-

4.5.5 CBIS Media 1 and 2

Media made from well water collected at depth during coring of the CBIS was used to make a media for general heterotrophic organisms. The well water used was from a depth of ~ 190 m. This depth lies within Microbiological Zone 1, in a region where indigenous microorganisms would be expected. However, as this depth is toward the top of the core in the marine deposits that rest on top of the CBIS, sediments from this area are not comparable to depths of interest within the CBIS. Yet, this sample of well water was all that was available, and it was useful to determine if it could serve as a more reliable culture media for subsurface microorganisms.

Media was inoculated with successful cultures from other depths to see if media derived from pore waters at depth would provide a better growth substrate for microorganisms than artificial media prepared in the laboratory. Ultimately, this was not the case. CBIS 1 media did not return any successful cultures, likely due to the high concentration of Na_2S used to maintain anaerobic conditions and the lack of nutrient sources, which may have been depleted by indigenous organisms during storage. Cultures were successful in CBIS 2 media, but because growth was limited compared to Het Med 1 further tests were not pursued.

4.5.6 Culturing in the CBIS Core

The complete results of all culturing media used to obtain indigenous microorganisms from CBIS cores can be found in **Table 20**. Cultures that could be replicated with better than 30% reliability were considered to be legitimate cultures from the deep subsurface environment of the CBIS. As expected, heterotrophic microorganisms were the easiest and most reliable cultures found in enrichments. Media for more specialized microorganisms, such as sulfate reducers and iron reducers, also provided successful enrichment cultures. Many of these specialized media yielded cultures at depths where chemical data would seem to suggest that the cultures are indeed composed of species targeted by the selected media. However, with the addition of CBIS core material required for inoculations, it is

possible that the enrichment cultures obtained are not composed of the target organisms, and instead are heterotrophic organisms capable of growing in the media provided. Further tests on these enrichment cultures were required in order to determine whether or not they could be confidently described as specialist microorganisms.

Table 20 – Culturing attempts at depths throughout the CBIS core and across all media types used. Hatched boxes indicate that no culturing was attempted at depth with the media. White boxes marked "-" indicate that all culturing attempts failed. Grey boxes indicate that cultures were successfully obtained. Numbers inside grey boxes indicate the percentage of successful cultures obtained in all replicate inoculations. When numbers are in parenthesis, this indicates that the cultures could not be reliably replicated and were therefore not considered as legitimate cultures for further study.

Sample Depth (Meters)	Het Med DH	Het Med 1	Het Med 1 Plates	Het Med 1 Aerobic	Het Med 1 Aerobic Plates	Humic Acid Utilisers	IR USGS	IR DH	IR Citrate Media	SR DH	SR Med 2	PM1	PM2	SR USGS	H2 Methano	A Methano	Fe Oxid Med
159.3		-	-							-							
250.5	(25)	(17)	-	-	-					-	-						
366.6	50	50	-	-	-					50	(17)						
441.7		-	-	-	-												
449.3		-	-	-	-					-							
544.3		(17)	-	-	-			-		-	-	67	-				
571.1		62	57	-	(14)			-		(25)	-	-	-				
588.1		(17)	-	-	-					-	-	50	50				
614.6		(17)	-	-	-					-							
643.5		75	71	-	-			-		-	-	-	33				
677.8		-	-	-	-	-	-			-					-	-	-
702.5						-	-								-	-	-
744.6		-	-			-	-	-		-					-	-	-
770.8						50	-								-	-	-
787.4						100	-								-	-	-
821.0						-	-								-	-	-
842.1		40	33	-	-												
844.6																	
899.2																	
901.0						-	-								-	-	
941.9	-	-	-	-	-			-		-							
997.7		-	-					-		-							
1085.5		-	-					-		-							
1419.1																	67
1447.5		(22)				-	100								-	-	-
1473.3		50	-	-				-		-							
1568.5	-	53	80	-	(14)			-		-							-
1608.8		62	71	-	(14)			-		-							-
1677.4																	-

In all, culturing results are in line with cell enumerations performed with DAPI (see

Chapter 3), and support the idea that three microbiological zones can be found within the

CBIS core (**Figure 36**). This indicates that sediments between the depths of 867 meters to

1096 meters were possibly sterilized by the impact and are still struggling to recover.

Additionally, inoculating sediments from this region with successful cultures obtained

from other depths in the CBIS indicates that, although sediments in Microbiological Zone

2 appear to contain little to no microorganisms, these sediments are indeed capable of

supporting microbial growth.
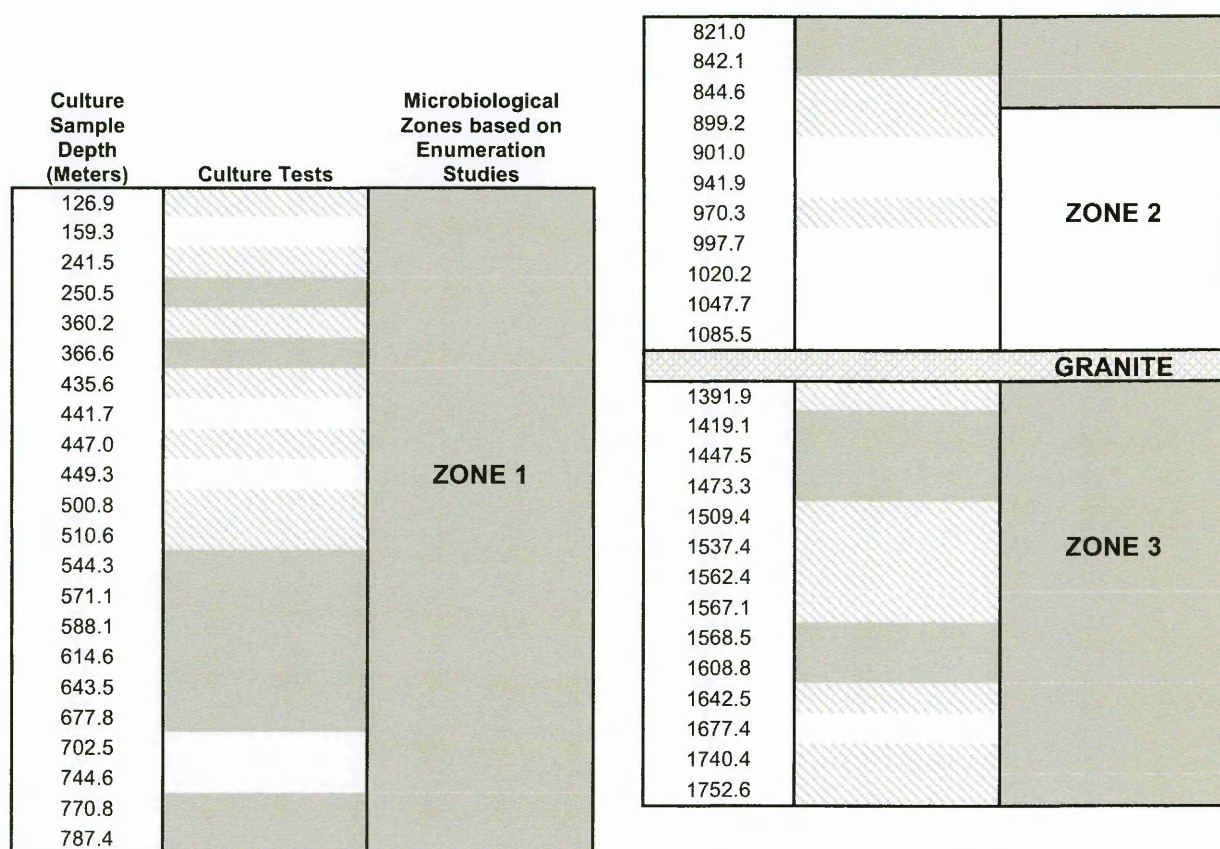
Figure 36 - Comparison of depths where successful cultures were obtained as related to the microbiological 'zones' defined by direct count cell enumeration studies. White boxes for Culture Tests indicate depths where culturing attempts were unsuccessful in all attempted media inoculations. White boxes for the Microbiological Zones represent depths where enumerations fell below the detectable limit. Grey boxes for Culture Tests indicate depths where cultures were successfully obtained by at least one of the inoculated media. Grey boxes for the Microbiological Zones indicate regions where cells were detectable in direct counts. Hatched boxes indicate depths where cell culturing was not attempted.

Successful cultures in Microbiological Zone 3 also support the idea that impact cratering

acts to provide new habitats for life in the deep subsurface, even though impacts can have

disastrous consequences for life at the Earth's surface. This region of the core is composed

of suevite, which is a form of impact breccia that contains both shocked and unshocked clasts as well as melt particles. This is thought to be material that was forced upward by the impact but was never ejected from the crater structure. Beneath the suevite and breccia rests schist and pegmatite, forms of metamorphic and igneous rock respectively. These materials also show indications of fracturing, fragmentation and rotation caused by the force of impact.

The materials at the bottom of the core show many indications that they were target materials, and as such would have been heated beyond sterilizing temperatures at the time of impact. The presence of cultures at these depths is an indication that re-colonization has occurred and, in fact, the processes of impact have generated new microhabitats for microorganismal communities. This could be through the creation of new pore spaces in which microorganisms can grow and also the redistribution of nutrients and carbon sources at depths where these vital inputs for life would otherwise be depleted.

4.6 SUMMARY

Enrichment cultures were obtained from a number of depths throughout the length of the core retrieved during the 2005 ICDP-USGS deep drilling of the CBIS. Successful culture depths correspond to microbiological Zones 1 and 3 as defined by cell enumeration estimates (**Chapter 3**) (**Figure 37**). Microbiological Zones 1 and 3 are depths where cell numbers were above the detectable limit. No cultures were obtained from microbiological Zone 2 with the methods employed in this study (**Chapter 3**). Zone 2 represents a region where cell numbers were also below the detectable limit (**Figure 37**). These results support the idea that microbiological Zone 2 is an impoverished region of the core, where numbers of cultureable microorganisms as well as total cell numbers are low.
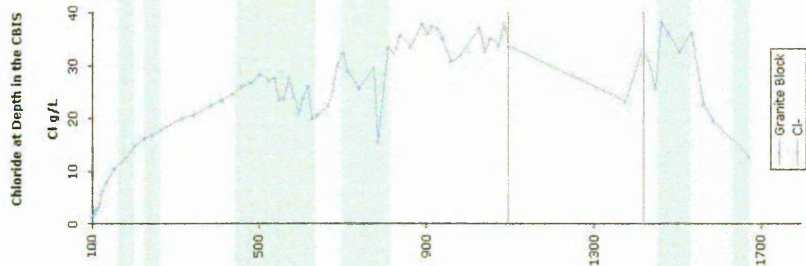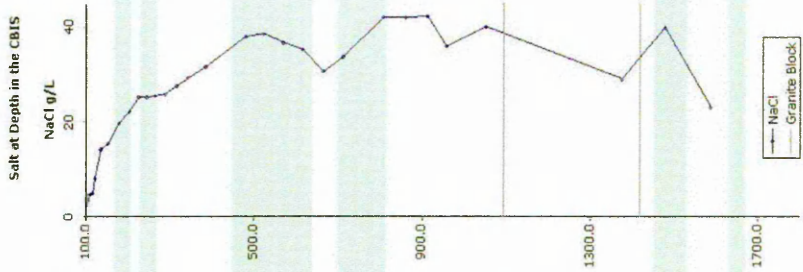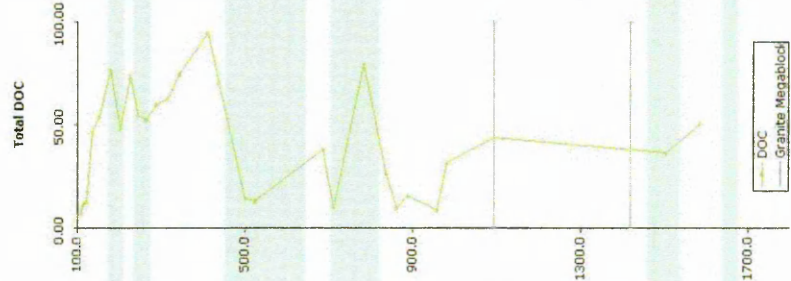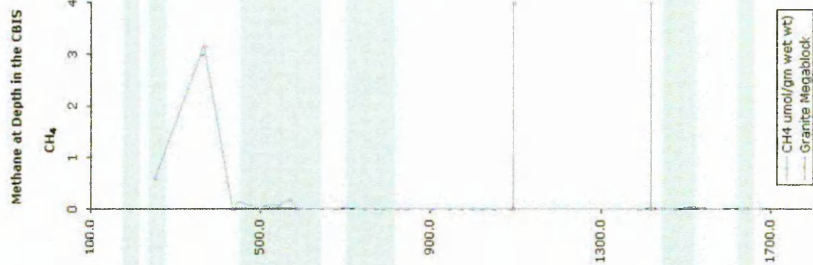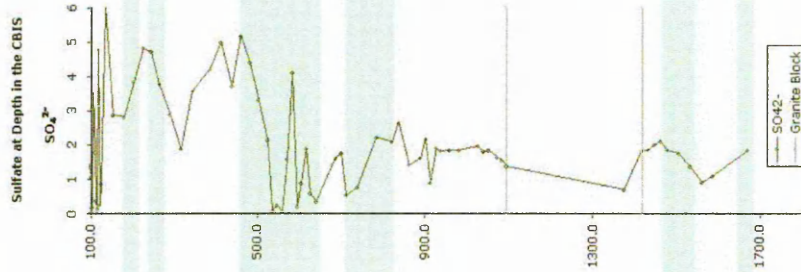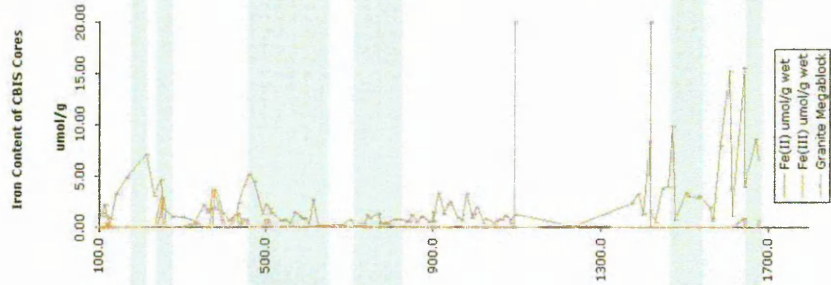
Figure 37 - Results of Enumeration and Culturing tests as related to porewater chemical data provided by the USGS. Green lines indicate regions where successful cultures were obtained. Grey lines indicate the region associated with the granite megablock. Red blocks for 'Culturing Results' signify depths where cultures were attempted but proved unsuccessful., while green boxes indicate successful cultures obtained with at least one media type.

The most robust cultures in laboratory conditions were obtained using media for heterotrophic organisms. Cultureable organisms were also obtained with media for iron reducers, sulfate reducers, iron oxidizers and humic acid utilisers. These more specialized organisms proved more difficult to grow and maintain in culture. Depths from which cultures were obtained could often be related to changes in the chemistry observed in porewaters (**Figure 37**).

Chapter 5: Obtaining and Characterizing Isolate Microorganisms from CBIS Core Samples

5.1 ABSTRACT

Attempts were made to isolate single species of microorganisms from successful enrichment cultures obtained from depths throughout the CBIS core. It was expected that with the unique environment of the CBIS, and the potential isolation of microbial communities within the crater sediments over geological time periods, the CBIS may represent a habitat for previously unidentified species or microorganisms that are related to, but distinct from, known types in the shallow subsurface. Heterotrophic isolates were obtained from Het Med 1 enrichment cultures at 571.1 m, 643.5 m, 1568.5 m and 1608.8 m. One isolate from Fe USGS enrichment cultures was obtained at a depth of 1447.5 m. Isolates were obtained through serial dilutions in liquid media and by plate spreading on solid media. All organisms were isolated in anaerobic conditions.

Once isolates were successfully obtained, a series of physiological and molecular studies were performed in an attempt to determine their species and classification. These included experiments for optimal NaCl concentration, pH, temperature and carbon source utilization. DNA was extracted from isolates and amplified using 16S rDNA primers. 16S rDNA sequences were obtained and potential isolates from each depth were compared to known species. Comparison of 16S rDNA sequences across isolate cultures was also used to confirm whether isolates obtained from enrichment cultures through different methods were identical species types or unique isolates from the communities present in enrichments.

Numerous problems were encountered in maintaining healthy isolate cultures, as the survival of organisms after being isolated from the enrichments was limited. Difficulty was also encountered in obtaining clean DNA that could be successfully amplified through PCR. Numerous methods for DNA extraction and varying conditions for amplification

were attempted in order to mitigate these problems; however, the troubles encountered with growth of isolates ultimately limited our ability to identify their species types with high confidence.

5.2 INTRODUCTION

Defining 'species' of microorganisms has been a much debated and ever evolving practice since the discovery of microorganisms only a few centuries ago. Microorganisms compose a major component of the Earth's biosphere, yet less that 5000 species had been defined by the beginning of the 21st century (Rosselló-Mora and Amann 2001). This low number arises from both the difficulties associated with isolating microorganisms in pure culture, and in the ways in which species of microorganisms are characterized.

As with higher eukaryotes, the original concept of 'species' was based on morphology. However, this concept was difficult to apply to microorganisms when they were first discovered only a few centuries ago. The small size and relative simplicity of cells in culture does not allow for a large amount of morphological variation. In fact, when microorganisms were first identified, researchers believe that there was only a single species and that it was able to develop into a variety of shapes (Wayne et al. 2001). Additionally, there is not a varied fossil record of microorganisms on Earth to define how speciation has occurred through time. It was not until phenotypic and genomic traits were used to classify microorganisms that a reliable taxonomic scheme could be developed (Wayne et al. 2001). The first modern identification key for microbial species was "Bergey's Manual of Determinative Bacteriology", published in 1923. This manual provided a framework of criteria so that microbiologists could use a unified system for nomenclature.

Since 1923, the number of methods for characterizing bacteria has constantly increased. Today, classifying species of microorganisms has developed beyond separating them into groups based on morphology viewed under the microscope to include other traits like the properties of DNA, rRNA sequencing and studying traits of chemical constituents

like amino acids, proteins and lipids (Wayne et al. 2001). Obtaining a pure culture of a microorganism is still a prerequisite for defining microbial species in a community. Individual organism types are still required in order to successfully perform biochemical tests, genome analysis or chemical component analysis. Pure cultures also allow for additional tests, such as an organism's tolerance to environmental factors like temperature and its ability to utilize various compounds for metabolism.

It is likely that the current practice of defining microbial species is conservative, and there is some argument in the literature that the species concept in microbiology is not comparable with concepts developed for higher eukaryotes (Wayne et al. 2001). The current method for defining microbial species is not perfect, but does provide reliable identification of strains (Stackebrandt and Goebel 1994).

5.2.1 Isolate Cultures from the CBIS

Efforts to culture microorganisms from CBIS core material using varied media types successfully returned a range of enrichment cultures from multiple depths in the core (see **Chapter 4**). The majority of these enrichment cultures were obtained with media for general heterotrophic organisms (Het Med 1). However, a select number of cultures were also obtained in media designed for organisms that utilize more specific metabolic pathways, such as iron reducers, iron oxidizers and sulfate reducers. Attempts to isolate individual species from each category of enrichment culture were performed.

Isolate cultures of individual species do not necessarily provide information on the microbial communities present in the environment as a whole. Typically, when isolating a single species from an enrichment culture, you are only able to obtain the most robust or dominant members of the community. Many organisms grow in a consortium and rely on other species in the community to survive and it is sometimes impossible to isolate such species as individuals. Additionally, communities contain members that are so reliant on their specific environment that they are unable to grow in the laboratory using traditional culturing methods.

However, isolate species do provide an opportunity to examine specific growth characteristics of species native to the target environment. Obtaining pure isolates is essential for accurate identification of individual species present in a community. In terms of the subsurface, many of the microorganisms present are unknown and uncharacterized. Because of the unique environment in which they live, some of these organisms have distinctive abilities in terms of the kinds of minerals they interact with and the methods they use to derive energy for growth. If such organisms can be successfully isolated, they may have many commercial uses in areas such as bio-mining or biological remediation for treating polluted soils. When isolates that participate in specific metabolic pathways, such as iron reduction, can be obtained, they can also serve to verify that specific media types were successful in obtaining their target communities in enrichment cultures.

5.3 METHODS

Standard methods for obtaining isolate cultures typically employ a series of dilutions from enrichment. When aliquots of an enrichment culture are inoculated in fresh media, competition between members in the community dictates that the most robust species are able to gain a foothold before less robust species, and subsequently out-compete other organisms for nutrients in the media. After a series of such dilutions, eventually only one species survives in culture.

5.3.1 Dilutions and Plate Isolation

Serial dilutions were performed in liquid culture from each enrichment culture obtained (see **Chapter 4**). Dilutions were made by cross-inoculation, where aliquots of liquid enrichment cultures were inoculated into fresh, sterile media. This process was repeated across further bottles of sterile media until no growth occurred. Eventually, after a series of dilutions, only one type of microorganism could be viewed under the microscope, indicating that a single species isolate culture had been obtained.

Spreading and streaking of bacterial colonies on plates was also used to obtain isolates from cultures. In this standard laboratory method, enrichment cultures were first

spread over the surface of solid media. If growth of colonies on the media surface

occurred, single colonies were removed by use of a sterile loop and streaked onto fresh

plates. Like the liquid dilution method, after a series of removing single colonies and

transferring them to new plates only a single species remains in successful colonies.

5.3.2 Most Probable Numbers

The most probably number (MPN) technique is a method developed to estimate the total

number of cultureable organisms present in an environmental sample, such as sediments.

Unlike direct cell counts (see **Chapter 3**), MPNs measure the ability of a culture to grow

in media based on qualitative properties of the microbial community (Oblinger and

Koburger 1975). For instance, if the object of a study is to estimate the number of

cultureable iron oxidizing organisms in a community, staining and counting cells under the

microscope will not be of use because stains like Acridine Orange and DAPI (see **Chapter

3**) are not specific to iron oxidizing bacteria. However, if cultures are grown in iron

oxidizing media it becomes easier to separate the target microorganisms for estimating

numbers.

MPN studies are performed by diluting and incubating cultures in liquid media and

through multiple rounds of serial dilutions. As a brief and general example, we'll look at an

MPN series made in 10 mL vials of liquid media. An aliquot of initial starting culture is

inoculated into fresh media at a 10x dilution (i.e. 1 mL of starting culture inoculated into 9

mL of sterile culture media) (**Figure 38**). An aliquot of this $1x10^{-1}$ dilution is then

inoculated into the next set of media, producing a $1x10^{-2}$ dilution (as compared to the

starting culture). As the initial starting culture is serially diluted (i.e. $1x10^{0}$, $1x10^{-1}$, $1x10^{-2}$,

$1x10^{-3}$...), fewer and fewer cells are present in the inoculums. Eventually, the dilution

becomes so great that no cells will be inoculated into the fresh media and no growth will

occur. This produces a pattern of successful growth in the dilution cultures (i.e.

successful/unsuccessful), and this pattern can then be used to estimate the total population

in the original culture based on mathematical formulae outlined by Halvorson and Ziegler (1933).

The MPN method has been used in microbiology studies for nearly a century, but there are limits to the population estimates they produce. This method assumes that cells in cultures are randomly and evenly distributed in the media, and that a single cell or more is capable of yielding a positive result in the dilution and media in which it is inoculated.
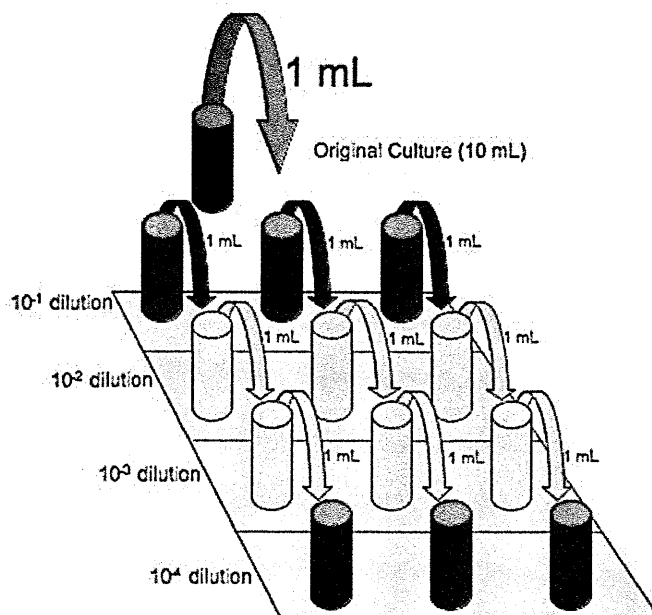
Figure 38 - Representation of MPN technique. One mL of the original culture is added to 9 mL of media producing a 1 in 10 dilution (10^{-1}). One mL of the 10^{-1} dilution is then transferred to 9 mL of media in Row 2 to produce 1 in 100 dilutions (10^{-2}). Dilution series continues until no growth is observed, indicating that no cells were transferred into the negative vials.

MPN serial dilutions can provide an estimate of living, cultureable organisms in a sample that are able to grow in a specific media type. However, MPNs also provide a simple way of obtaining isolated individuals from environmental samples. Like standard serial dilutions, the final successful dilution in MPN studies often produces a single species type in culture.

Starting inoculations of media were made by adding sediment from CBIS core samples to 10 mL of media. Depths for CBIS core samples were based on those that

produced enrichment cultures by similar methods (see **Chapter 4**). Dilutions were then made by transferring 1 mL of enrichment culture into 9 mL of sterile growth media. Further dilutions were then performed as discussed above until no growth was observed in the final dilutions.

MPN studies were performed using Het Med 1, which was the most successful media for producing enrichment cultures (see **Chapter 4**). MPNs with Het Med 1 were performed on core samples 5, 8, 16, 19, 45 and 46. Dilutions were prepared using 10 mL of media in pre-autoclaved 15 mL Hungate tubes sealed with rubber stoppers and gassed with N_2. All media preparations were as previously discussed for enrichment cultures (see **Chapter 4**). Inoculations were incubated at 37°C. Growth in dilutions was verified by epifluorescence microscopy using DAPI, Syto 9 and Sybr Green I staining (see **Chapter 3** for information on these dyes). Isolates were then collected from the final dilutions in each series.

5.3.3 Epifluorescence Microscopy

Microorganism cultures were viewed with the light microscope (Leica DMRP) to characterize cell phenotypes. For visualization by epifluorescence microscopy, cells were stained using the DNA-binding dyes DAPI (at a concentration of 5 μL/mL), Syto 9 (3 μL/mL) or Sybr Green I (5 μL/mL) (see **Chapter 3** for details on dyes). Direct aliquots of liquid cultures were used for viewing. In cases where cell densities were extremely low, and cell counts were not being performed, a 1 mL aliquot would be spun down by gentle centrifugation (~30 seconds at 3000 rpm) in order to increase the amount of cells per field of view. For plate cultures, colonies were picked using either a pre-autoclaved sterile toothpick or a sterile plastic loop and re-suspended in 500μL filter-sterilized PBS solution. All slides were prepared with 10 μL of suspended cells on glass slides under a coverslip.

Growth of cultures in dilutions and in optimal growth tests (see below) were also identified using epifluorescence microscopy by staining aliquots of cultures using the

DNA-binding dyes DAPI and Syto 9 (at a concentration of 3 µL/mL) (see **Chapter 3** for

details on dyes). Cell counts across 50 fields of view were performed in order to build

growth curves over the duration of incubation periods.

5.3.4 Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) was performed with the aid of Heather A.

Davies, Manager of the EM Suite, Open University. TEM samples were prepared from

both liquid and plate cultures using a fixative prepared as: 2.5% glutaraldehyde and 0.1%

ruthenium red in 0.1 sodium cacodylate at pH 7.2 (cacod/RR, Agar Scientific, Stansted,

England).

Liquid cultures were prepared by first centrifuging 0.5 to 1 mL of liquid culture to

pellet cells. The pellet was then re-suspended in the fixative (above) and fixed for one hour

at room temperature.

Processing and Thin Sectioning

Processing and thin sectioning of samples was performed by Heather A. Davies, Manager

of the EM Suite, Open University. All processing of samples was achieved by

microcentrifugation between each step. Samples were first washed by aspiration in a buffer

solution and post-fixed in 2% osmium tetroxide in cacod/RR for one hour at room

temperature. Samples were then washed again and dehydrated through a graded series of

acetone (30%, 50%, 70% and 90%). After three changes in 100% acetone, samples were

then infiltrated with acetone using Epon resin (1:1) overnight followed by neat Epon for

five hours. Liquid samples were then embedded in microcentrifuge tubes, while solid

culture samples were embedded in BEEM 000 capsules. Polymerisation of the resin was

performed over a 48 hour period at 60°C.

Samples embedded in blocks were sectioned using a Leica UCT ultramicrotome.

The resulting 70nm sections were placed on Formvar® carbon-filmed copper slot grids

(Structure Probe, Inc.). The sections were then counter-stained with 4% uranyl acetate and

lead citrate (Renolds 1963).

TEM Imaging

Counter-stained 70 mm sections were examined in a JEM 1400 (JEOL) transmission electron microscope at 80kV with the aid of Heather A. Davies, Manager of the EM Suite, Open University. Digital images of culture samples were obtained using an AMT XR60 digital camera with a resolution of 11 megapixels.

5.3.5 Molecular Biology Analysis

Phenotypic tests alone cannot be used to identify microorganisms in an environment (Jiménez 1990), and modern studies on microbial communities also require molecular tests to define the genetic relationships between species present in an environment. DNA was extracted from isolate cultures using a selection of methods including Freeze-thaw lysis (see below) and commercial DNA extraction kits including: FastDNA® Spin Kit (MP Biomedicals), the QIAgen Puregene Tissue kit (QIAGen), the UltraClean™ Soil DNA Isolation Kit (MoBio Laboratories, Inc.), PowerMax Soil DNA Isolation Kit (MoBio) and the QIAamp DNA stool mini kit (QIAGen). DNA extractions were performed in the EGL laboratories at the Open University, UK, or during visitations to the Voytek Microbiology Laboratories at the USGS in Reston, VA. Extracted DNA was then amplified by polymerase chain reaction (PCR) using 16S rDNA primers and gel purified by electrophoresis.

In some cases, cultures were brought to the Voytek Microbiology Laboratory at the USGS in Reston, VA, for DNA extraction and PCR amplification. Bacterial DNA was amplified using the polymerase chain reaction (PCR) in a Perkin Elmer Geneamp 2400 thermal cycler with 16S rDNA primers (Brunk *et al.* 1996; Lane 1991). A variety of primers were used for various cultures until one set produced successful products (**Table 21**), producing partial 16S rDNA gene sequences. The conditions for 16S rDNA PCR (30 cycles) were: denaturing at 94 °C (30s), annealing at 56 °C (30s), and extension at 72 °C (1m). At the USGS, PCR amplicons were purified using the Wizard PCR purification kit (Promega, Madison, WI). Cycle sequencing of both strands of the DNA product was

performed at the USGS in Reston, VA, using big dye v3.1 (Applied Biosystems, Foster City, CA) and run on an ABI310 genetic analyzer. Sequences were edited and assembled using Autoassembler.

Table 21 - List of primers used for PCR amplifications of DNA extracted from CBIS cultures.

Primer[a]	Target	Sequence (5' to 3')	Reference
pA	16S-Eubacteria	AGA GTT TGA TCC TGG CTC AG	Bruce et al, 1992 (AEM, 58:3413-3416)
pH	16S-Eubacteria	AAG GAG GTG ATC CAG CCG CA	Bruce et al, 1992 (AEM, 58:3413-3416)
Com1	16S-Eubacteria	CAG CAG CCG CGG TAA TAC	Schwieger and Tebbe, 1998 (AEM, 64:4870-4876)
Com2	16S-Eubacteria	CCG TCA ATT CCT TTG AGT TT	Schwieger and Tebbe, 1998 (AEM, 64:4870-4876)
27f	16S-Eubacteria	AGAGTTTGATCMTGGCTCAG	Lane 1991
43f	16S-Eubacteria	TCAGA(A/T)(C/T)GAACGCTGGCGG	van der Gast and Thompson 2004
63f	16S-Eubacteria	CAG GCC TAA CAC ATG CAA GTC	Marchesi et al. 1998; 2001
519f	16S-Eubacteria	GTATTACCGCGGCTGGCTG	Lane et al. 1985
1100r	16S-Eubacteria	GGGTTGCGCTCGTTG	Lane 1991
1387r	16S-Eubacteria	CRTGTGTGGCGGGCA	Lane 1991
1392r		CGGAACATGTGMGGCGGG	Stahl and Amann 1991
1492r	16S-Eubacteria	TACGGYTACCTTGTTACGACTT	Lane 1991
Arch 671f	16S-Archaebacteria	GGCCCTACGGGGSGCASCAGGCGC	Giovannoni et al. 1990; Brunk et al. 1996
Arch 15256r	16S-Archaebacteria	AATTGGAKTCAACGCCGGR	DeLong 1992; Brunk et al. 1996
Geo494f	Highly conserved genes in the iron reducer Geobacter	AGG AAG CAC CGG CTA ACT CC	Holmes et al., 2002
Geo825r	Highly conserved genes in the iron reducer Geobacter	TAC CCG CRA CAC CTA GT	Holmes et al., 2002
SbrF	Not available	Not available	Provided by the Voytek Microbiology Laboratory, USGS, Reston, VA.
SbrR	Not available	Not available	Provided by the Voytek Microbiology Laboratory, USGS, Reston, VA.

Freeze-Thaw Lysis

The freeze-thaw lysis (FT) method of DNA extraction is relatively simple when compared to other techniques, and has proven effective in obtaining DNA from cell cultures in numerous studies. Due to the ease and low-cost, the FT method is a standard technique used in molecular studies and often accompanies other chemical lysis techniques used in the laboratory in order to increase DNA yield (Miller et al. 1999).

When living cells are frozen and thawed, the formation of ice crystals in the medium and within the cells themselves can cause cells to rupture. This frees DNA from the cells and into solution where it can be accessed and then amplified using PCR.

The first attempt at FT DNA extraction from CBIS isolate cultures was performed on eleven cultures obtained from varying depths throughout the core (**Table 22**).

Table 22 Cultures used in FT DNA extraction Test 1.

Label		Culture	Depth (m)	Notes
A	19	CBIS 8 Fe-Citrate E	366.6	Enrichment culture in Fe Citrate Medium
A	29	CBIS 16 I2	571.1	Optimal pH experiment using CBIS 16 Isolate 1.
A	2	CBIS 16 PI	571.1	Plate Isolate transferred to liquid culture
A	3	CBIS 19 I1	643.5	CBIS 19 I1 in liquid culture
A	17	CBIS 19 I1	643.5	CBIS 19 I1 culture taken from tests for optimum [NaCl]: 100 g/L
A	39	CBIS 19 I1	643.5	CBIS 19 Isolate 1 culture taken from tests for optimum [NaCl]: 45 g/L
A	8	CBIS 19 PI	643.5	Plate Isolate transferred to liquid culture
A	1	CBIS 39-1 E	1447.5	Fe Reducer from Fe USGS Medium
A	22	CBIS 45 I1	1568.6	Isolate obtained through consecutive liquid dilutions
A	7	CBIS 46 I1	1608.8	Plate Isolate transferred to liquid culture
A	6	CBIS 46 PI	1608.8	Plate Isolate transferred to liquid culture

Actively growing cell cultures were selected for DNA extraction. Aliquots of 200 μL from each culture were placed in pre-autoclaved plastic Eppendorf tubes. Cultures were then agitated for fifteen seconds by vortexing and placed in a -20°C freezer overnight. The following day, samples were thawed and vortexed again for fifteen seconds before addition to the PCR mix containing bacterial pA/pH primers, 5x MasterTaq, 10X Buffer with Mg^{2+}, dNTP (10mM) and Ultrapure sterile H_2O. Denaturing of DNA and enzyme activation was performed by heating samples to 94°C for 10 minutes. Thirty-five cycles for PCR were then performed, consisting of:

4. Denaturing Step: 94°C at 1 min

5. Annealing Step: 55°C at 1 min

6. Extending Step: 72°C at 1 min (5 min for the final cycle)

Samples were then run on an agarose gel and stained using ethidium bromide to confirm the presence of PCR product. When the NanoDrop spectrophotometer (Thermo Scientific) became available in the lab in the summer of 2008, DNA content per mL was estimated to verify successful DNA extractions (See below).

Direct PCR from Plate Colonies

PCR was also performed directly from colonies grown on agar plates. For these reactions, a single colony was added to two separate PCR mix containing either the enzyme MasterTaq Polymerase (Eppendorf), or the enzyme Red HotTaq Polymerase (ABgene). Both mixes contained the primers pA and pH' (Bruce et al. 1992) (**Table 21**). For each PCR reaction, one blank mix that contained no cells was also run to check for the absence of contaminants in the PCR mixture. PCR mixtures were UV-treated for 10 minutes prior to addition of the Taq enzymes and cell colonies. There is a risk of damaging primers when exposing them to UV; however UV treatment was needed to ensure there was no contamination in the mixture. All preparation of PCR mixtures was performed in the laminar flow hood following cleaning of surfaces with ethanol. Cell lysis was performed by an initial heating step at 94°C for 15 minutes, and thermal cycling was performed using 30 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, followed by an elongation step of 72°C for 10 minutes.

Table 23 - PCR Mix 1 using Red HotTaq Polymerase (ABgene)

	Volume (μl) for 1 PCR reaction	Final volume
Primer PA (10μM)	5	1 μM
Primer PH (10μM)	5	1 μM
MgCl2 (25mM)	3	1.5mM
Buffer 10X without Mg^{2+}	5	1X
dNTP (10mM)	1	200 μM
Taq Polymerase (5U/μl)	0,5	5 U
Ultrapure sterile water	30.5	Final vol = 50 μl

Table 24 - PCR Mix using MasterTaq Polymerase (Eppendorf)

	Volume (μl) for 1 PCR reaction	Final volume
Primer PA (10μM)	5	1 μM
Primer PH (10μM)	5	1 μM
5X TaqMaster (heating at 60°C until complete dissolution before using)	10	1X
Buffer 10X with Mg^{2+}	5	1X
dNTP (10mM)	1	200 μM
Taq Polymerase (5U/μl)	0,5	5 U
Ultrapure sterile water	23.5	Final vol = 50 μl

An 8µl aliquot of each PCR products was then run on a 0.8% agarose gel alongside a 1Kb ladder (Invitrogen). After running, the gel was introduced into an ethidium bromide bath for 15 minutes and washed in water for 10 minutes. Gels were then viewed under UV to identify successful PCR products. An aliquot (8µl) of each PCR product (with 2.5µl of loading buffer) was then run on a 0.8% agarose gel.

Additional sequencing of plate colonies was performed at the USGS in Reston, VA. For these reactions, single plate colonies were picked and added to a solution of Te Buffer (**Appendix III**) in pre-autoclaved Eppendorf tubes. Colonies were then shipped to the Voytek Microbiology Laboratories at the USGS for DNA extraction, PCR amplification and sequencing.

Extractions from Enrichment Cultures
Because many of the enrichment cultures obtained from CBIS sediments appeared to be composed of single cell-types, DNA extraction was performed on enrichment cultures and DNA was then amplified and sequenced directly in order to determine if a clean 16S rDNA sequence could be obtained. DNA was extracted using the QIAgen puregene tissue kit (QIAGen). Extraction, PCR amplification and sequencing were performed during a research visit to the Voytek Microbiology Labs at the USGS in Reston, VA, as described above.

DNA Concentration Estimates
The concentration of DNA in solution obtained from the various methods of DNA extraction used for cultures was determined by use of a NanoDrop 1000 Spectrophotometer (Thermo Scientific). DNA content was estimated based on 1.5 µL aliquots as per the manufacturer's instructions.

G+C Content
The content of the nucleic acids Guanine and Cytosine 16S rDNA sequences was determined using the Cumulative G+C Profile method (Zhang and Zhang 2008), and using the Z Curve Database of Genomes (http://tubic.tju.edu.cn/zcurve/) provided by the Tianjin

University BioInformatics Centre. Additionally, G+C content and estimated melting temperature for some samples was calculated using MacVector ™ 6.0.

Because the total genomic sequence of organisms was not used, this provided only an estimate of the total G+C content based on the partial 16S rDNA sequence. G+C content is estimated by taking the total number of Guanine and Cytosine base pairs as compared to Adenine and Thymine pairs in a sequence of DNA. There is a correlation between G+C content and phylogeny in microorganisms, and determining G+C content can thus be used to aid in the identification of organism types (Muto and Osaka 1987). For this reason, G+C content has become a standard piece of evidence used in characterizing new species of microorganisms. G+C content of an organisms' DNA can be compared to databases, such as the NCBI Taxonomy browser (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Undef&id=201174 &lvl=3&lin=f&keep=1&srchmode=1&unlock, accessed 8/10/08) to provide information on the potential identity of isolates.

G+C content estimates based on 16S rDNA sequences have only a weak correlation with total genomic G+C content (Muto and Osaka 1987). Unfortunately, methods available for estimating G+C within the total genomic DNA of a microorganism require growth of dense cultures of the organism to provide enough cellular material for G+C to be determined via the melting point of DNA (Jiménez 1990). Microorganism cultures from the CBIS did not grow in dense cultures even after a year of cultivation in some cases, and not enough cellular material could be collected in order to obtain full G+C estimates.

5.3.6 OPTIMAL GROWTH TESTS

Further studies on the optimal conditions for growth were performed on three of the isolates obtained from the CBIS. This included determining the optimal concentrations of NaCl, optimal pH, temperature and carbon source utilization profiles. Because optimization tests require isolates to be re-grown across varying media conditions, the most robust isolates were selected for these studies.

Optimal NaCl Concentration

The optimal concentration of NaCl was determined for isolates by preparing culture media with NaCl ranging from 0 g/L to 100 g/L. Concentrations of NaCl in the CBIS core ranged from ~ 8 g/L to ~ 44 g/L. The majority of CBIS cultures were obtained using NaCl concentrations near the native concentration at depth for the core materials, which was in the range of 16 g/L to 36 g/L. It was thought that varying NaCl from 0 g/L to 100 g/L would provide a wide enough range to determine differences in growth rates between replicate cultures.

Media was prepared in 15 mL Hungate tubes, sealed with rubber stoppers and gassed with N_2. Three replicate cultures were prepared for each concentration tested as well as one un-inoculated, sterile control. All replicates were inoculated from a single isolate culture and starting cell numbers were determined by epifluorescent cell counts. Cultures were then incubated at 37°C and cell counts were prepared on two of the three replicates at each concentration over the course of incubation. A final ending cell count was made on all replicates at the completion of the experiment.

Optimal pH

The pH through the CBIS core ranges from 6.8 to 8.4, with an average pH in samples of ~7.46 (data provided by the USGS and is not shown). As with NaCl, optimal pH experiments were set up using three replicates and one control with pH in the range of 3.0, 6.0, 7.5, 9.0 and 12.0. Cultures were prepared in 15 mL Hungate tubes sealed with rubber stoppers and gassed with N_2. In some cases, cultures were also prepared in 30 mL aluminium-cap anaerobic bottles. Media were prepared following the standard procedure and then adjusted to the varying pH values by titrating either sterile 10 M NaOH or 10 M HCl into solution. Cell counts were performed under the microscope to monitor growth using DAPI or Syto 9. When samples for cell counts were taken from cultures, a second sample of the media was taken to monitor pH by use of indicator test strips.

Optimal Temperature

Tests for optimal growth temperature were prepared in 15 mL Hungate tubes sealed with rubber stoppers and gassed with N_2. Three replicates and one control were prepared at each temperature. Cultures were refrigerated at 4°C and incubated at 27°C, 37°C and 50°C. One set of replicates was also autoclaved at 120°C for 30 minutes.

Carbon Source Utilization Profiles

Carbon source utilization profiles were constructed for isolates by use of the Biolog-AN Microplates (Biolog Inc., Hayward, California). Isolates were removed from growing culture by pelleting cells gently in a 7 cm (diameter) centrifuge for 3 minutes at 2000 rpm. Cells were then re-suspended in a sterile, anaerobic solution of Het Med 1 with all carbon sources removed. NaCl was adjusted to a concentration equivalent to that present in the native environment at depth for the selected isolates. Plates were then inoculated by adding 100 μL of suspended cells into each well. Plates were then incubated at 37°C and monitored every two days for three weeks.

5.4 RESULTS

The majority of isolates obtained from CBIS cultures were heterotrophic organisms grown in Het Med 1 media (See **Chapter 4**). The enrichment cultures obtained with Het Med 1 were the most robust under laboratory conditions, and showed the most consistent growth when dilutions were made. However, even cultures from Het Med 1 would not survive through many separate dilutions, and most showed no growth after just two or three cross inoculations. Similarly, with isolates obtained on solid media, cultures were often difficult to transfer to fresh plates after only one or two attempts at picking colonies and streaking onto new media.

5.4.1 Most Probable Numbers

Most probable number (MPN) studies were performed on five depths in the CBIS. Depths were chosen based on their ability to yield enrichment cultures using the heterotrophic media, Het Med 1, and correspond to enrichment cultures CBIS 5E, CBIS 8E, CBIS 16E,

CBIS 45E and CBIS 46E. An initial enrichment culture was prepared by adding sediment

to 10 mL Het Med 1 in Hungate tubes sealed with rubber stoppers and gassed with N_2.

Serial dilutions were then prepared with three tubes at each dilution as described above.

MPN estimates were made based on positive/negative growth of cultures using the

standard MPN table for dilution with three tubes provided by the US Food and Drug

Administration (http://www.cfsan.fda.gov/). The results of MPN studies and the estimated

number of cultureable cells per gram of sediment are shown in **Table 25**.

Table 25 - Results of MPN studies at the depths tested. Those marked N/A indicate attempts where MPN tests failed to produce useable results. DAPI counts are presented from the closest depth (above or below) where enumeration samples were collected and counted.

Depth (m)	Corrspnd Cultures	Estimate Cells per g sediment	Sediment in initial tube (g)	Adjusted for initial amount of sediment	DAPI Counts (nearest core)	MPN % of total DAPI counts
250.5	CBIS 5E	N/A	0.142	N/A	6.09×10^6	N/A
366.6	CBIS 8E	3.6×10^0	0.179	2.0×10^1	1.08×10^7	0.000001
571.1	CBIS 16E	9.3×10^2	0.1852	5.0×10^3	4.88×10^4	0.1
1568.5	CBIS 45E	7.5×10^2	0.6233	1.2×10^3	3.17×10^5	0.004
1608.8	CBIS 46E	1.5×10^3	0.1497	1.0×10^4	2.19×10^5	0.05

CBIS 5E – 250.5 meters

Although enrichment cultures from a depth of 250.5 m (CBIS 5E) showed only minimal

growth and cultures were difficult to maintain, most probable number tests were attempted

with sediment from this depth. MPN experiments were begun with 0.142 g of sediment in

10 mL of Het Med 1 in a Hungate tube sealed with a rubber stopper. Three replicate lanes

of ten dilutions from 10^{-1} to 10^{-10} were made. Cells were initially identified in the starting

inoculation after 14 days, but died off after ~30 days of incubation. Dilution cultures in

Hungate tubes were checked regularly for growth. However, no dilutions showed any

indication of growth, even after 360 days of incubation.

CBIS 8E – 366.6 meters

As with depths of 250.5 m, cultures obtained with sediments from 366.6 m (CBIS 8E)

were difficult to maintain under laboratory conditions and showed minimal and sporadic

growth. MPN experiments were begun with 0.179 g of sediment in 10 mL of Het Med 1 in

a Hungate tube sealed with a rubber stopper. Three replicate lanes of tubes were prepared

at eight dilutions ranging from 10^{-1} to 10^{-8}. Cells were initially present in the starting

inoculation, but died off after ~30 days of incubation. Positive growth of cultures in

dilutions was found after 330 days of incubation. At the completion of the experiment,

MPN tests showed that the estimated number of cultureable cells with Het Med 1 present

in sediments at 366.6 m was 5.1×10^{1} cells/g (**Table 25**).

10^{0} 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8}
Original
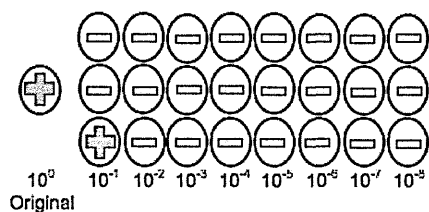
Figure 39 - Positive/Negative results for MPN tests from sediment at 366.6 m. Plus signs represent tubes where cells were successfully grown. Negative signs represent tubes where no growth was seen.

CBIS 16E – 571.1 meters

MPN studies on enrichment cultures from 571.1 m (corresponding to enrichment cultures

CBIS 16E) showed that sediments from this depth contained roughly 5.9×10^{3} cultureable

cells with Het Med 1 per gram of sediment. Results of the MPN test can be seen in **Figure**

40. Final dilutions in each replicate lane were then re-inoculated in fresh Het Med 1 and

examined under the microscope to see if multiple cell morphologies were present. As with

the original enrichment culture, all cells appeared to be similar. DNA was then extracted

from these enrichment cultures in order to see if clean sequences could be obtained and if

they varied from sequences from isolates obtained by cross-inoculations and plate cultures.

Ultimately, no amplifiable DNA was successfully obtained from any of the extraction

techniques, severely limiting the ability to accurately identify the isolates present at this

depth (See below).

10^{0} 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8}
Original
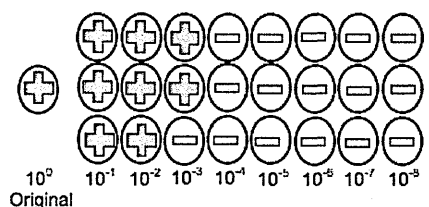
Figure 40 - Positive/Negative results for MPN tests from sediment at 571.1 m. Plus signs represent tubes where cells were successfully grown. Negative signs represent tubes where no growth was seen.

CBIS 45E – 1568.5 meters

Sediments from a depth of 1568.5 m were prepared as above at dilution ranging from 10^{-1} to 10^{-7}. As with other MPN tests, the final dilutions in each replicate lane were then inoculated into fresh Het Med 1 at the completion of the experiment and examined to see if isolates had potentially been obtained. Cultures from lane one and two appeared to be identical to the coccoid cells seen in the original enrichment cultures of CBIS 45E. The culture from lane three, however, appeared to be of cells not seen in CBIS 45E (**Figure 58**). DNA extractions were attempted on these samples to identify potential species types. As with CBIS 16 isolates above, amplifiable DNA from CBIS 45 isolates proved difficult to obtain.
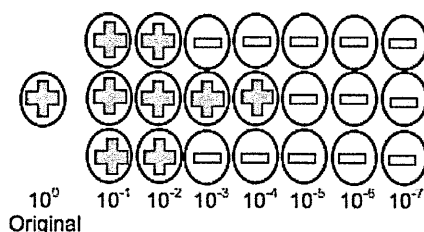
Figure 41 - Positive/Negative results for MPN tests from sediment at 1568.5 m. Plus signs represent tubes where cells were successfully grown. Negative signs represent tubes where no growth was seen.

CBIS 46E – 1608.8 meters

Sediments from a depth of 1608.8 m were prepared at dilutions ranging from 10^{-1} to 10^{-8}.

At the completion of the experiment, the final dilutions in each replicate lane were again removed and examined. All cultures appeared to be composed of the same cell types. These cultures were stored as potential isolates for further examination.
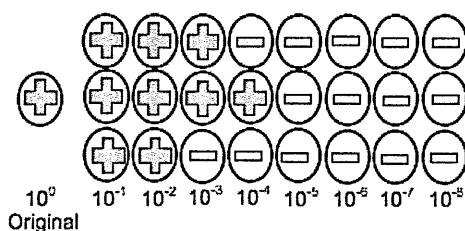
Figure 42 - Positive/Negative results for MPN tests from sediment at 1608.8 m. Plus signs represent tubes where cells were successfully grown. Negative signs represent tubes where no growth was seen.

5.4.2 Isolates Obtained Through Serial Dilutions in Liquid Media

Serial dilutions were performed with all successful enrichment cultures obtained from CBIS sediments. For the enrichment cultures CBIS 5E (250.5 m), no growth occurred in dilutions from the original enrichment culture. Only one dilution from CBIS 8E (366.6 m) could be obtained, and it was taken from the final successful dilution in MPN tests (see above). Additional isolates were obtained through dilutions of other enrichment cultures obtained with Het Med 1, which showed more consistent growth in the laboratory than CBIS 5E and CBIS 8E. Isolates that were successfully obtained could often not be grown on solid media, and would only show growth in liquid. Additionally, once isolated, it was very difficult to successfully transfer isolates to fresh media. In order to maintain isolate cultures, freshly prepared sterile media had to be added to culture bottles to restore nutrients.

CBIS 16 Enrichment - 571.1 m

Isolates were obtained from CBIS 16E (571.1 m) through serial dilutions. The first isolate obtained, dubbed CBIS 16I1, was grown after direct dilutions from the original CBIS 16E culture. The slender, filamentous cells of CBIS 16I1 typically clumped closely together to form mats and could rarely be seen as free-floating individual cells (**Figure 43**).
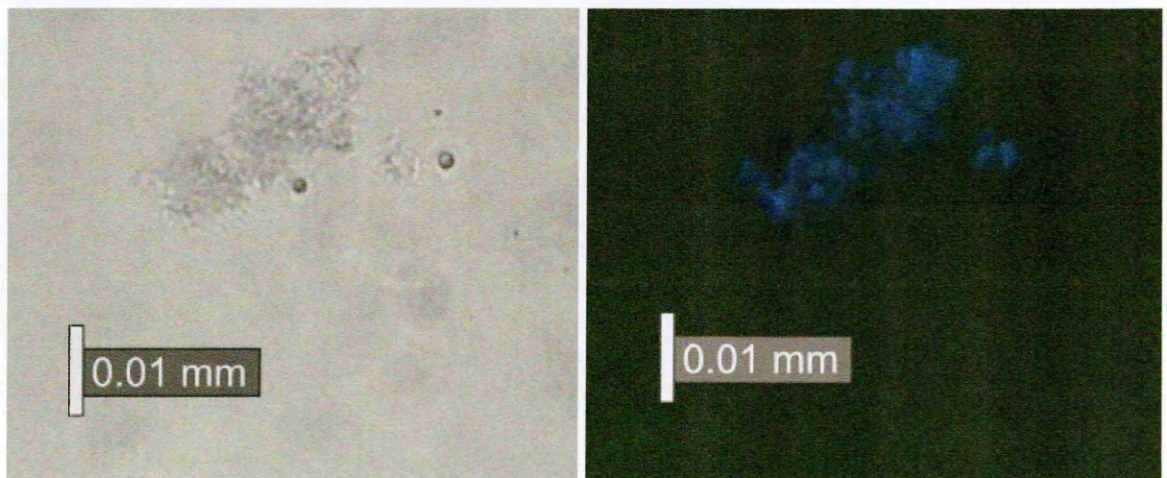
Figure 43 - CBIS 16 Isolate 1 (CBIS 16 I1) from the enrichment culture CBIS 16E obtained from sediments at 571.1 m. Images are shown in white light (left) and stained with the fluorescent dye DAPI (right).

A second isolate was collected from dilution series of CBIS 16E and dubbed CBIS 16I2. This second isolate appeared similar to CBIS 16I1, with a similar size and morphology. Cells grew as filamentous strands (**Figure 44**), often clumping together in large mats (**Figure 45**).
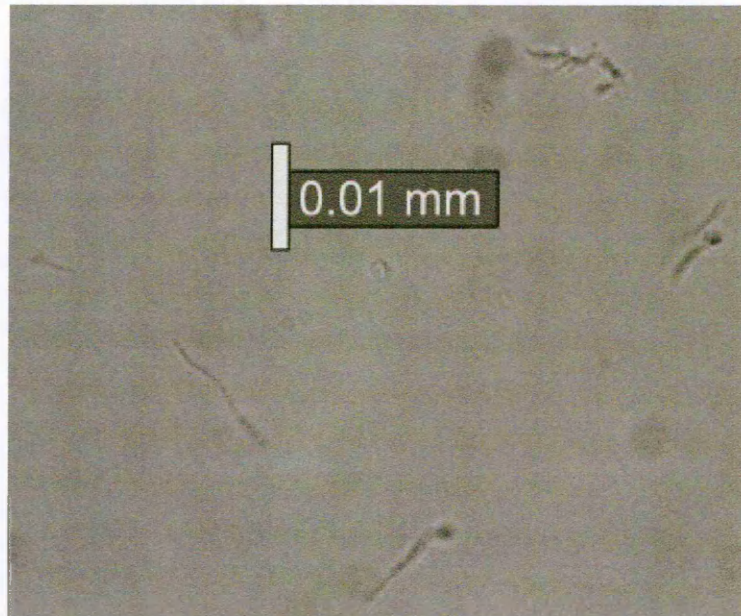
Figure 44 - Individual, filamentous strand-like cells of CBIS 16I2, obtained from dilution series of enrichment cultures CBIS 16E associated with sediments at a depth of 571.1 m.
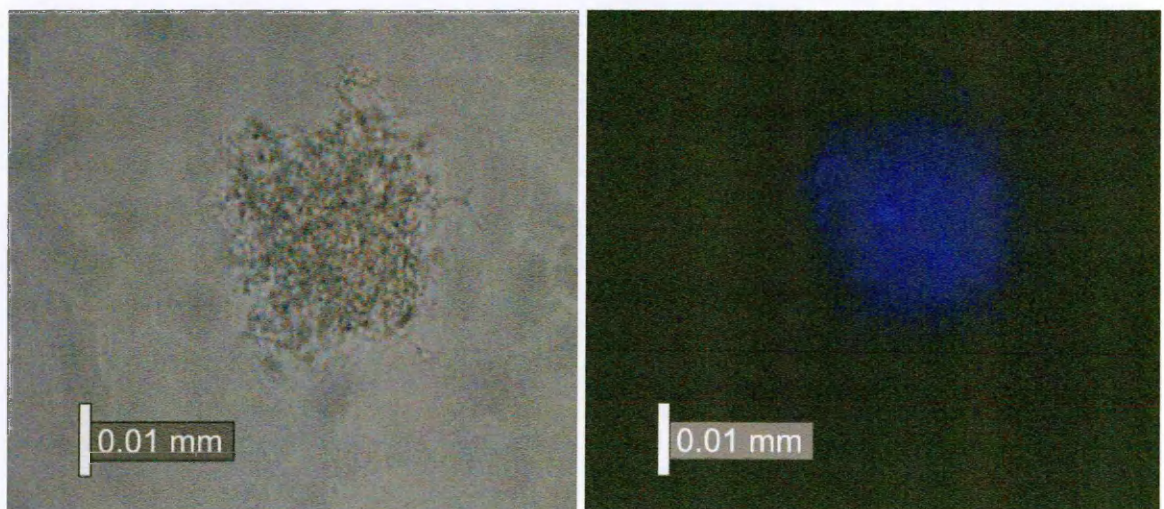
Figure 45 - CBIS 16 I2 clumping after long periods of incubation, shown in white light (left) and stained with the fluorescent dye DAPI (right).

The strand-like cells of CBIS 16I2 were sometimes small contained few (or no) branches (i.e. **Figure 47).** However, CBIS 16I2 cells were most often seen with extensive branches (i.e. **Figure 46**). Staining of cells showed distinctive 'pockets' of fluorescing DNA (**Figure**

46), indicating that these branching structures may actually be composed of multiple, attached cells.
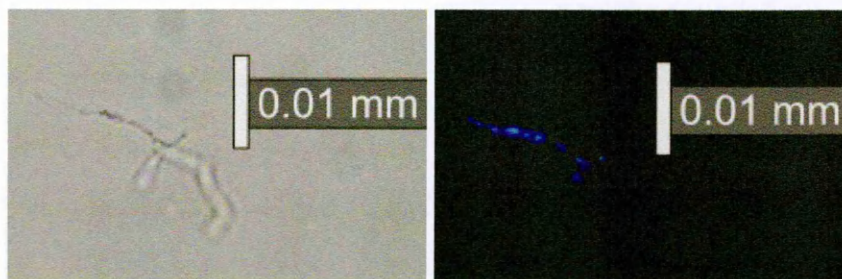
Figure 46 - Branching strands of CBIS 16I2 in white light (left) and stained with DAPI (right).
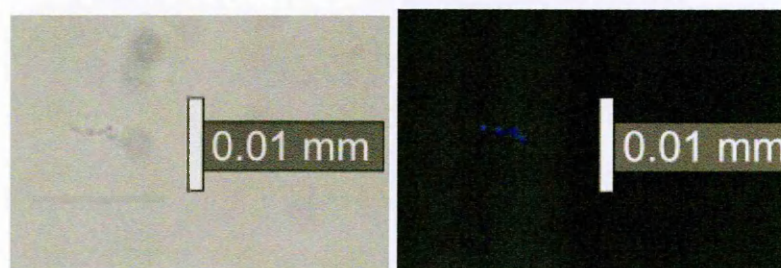
Figure 47 - A small branching strand of CBIS 16 I2 in white light (left) and stained with DAPI (right).

CBIS 16 I3 was a culture retrieved from MPN experiments performed on sediments collected at 571.1 m. CBIS 16 I3 was the only isolate removed from MPN experiments that could then be re-grown in liquid media. Cells clumped in groups similar to other CBIS 16 isolates, however clumps rarely exceeded more than ~15 nm in width.
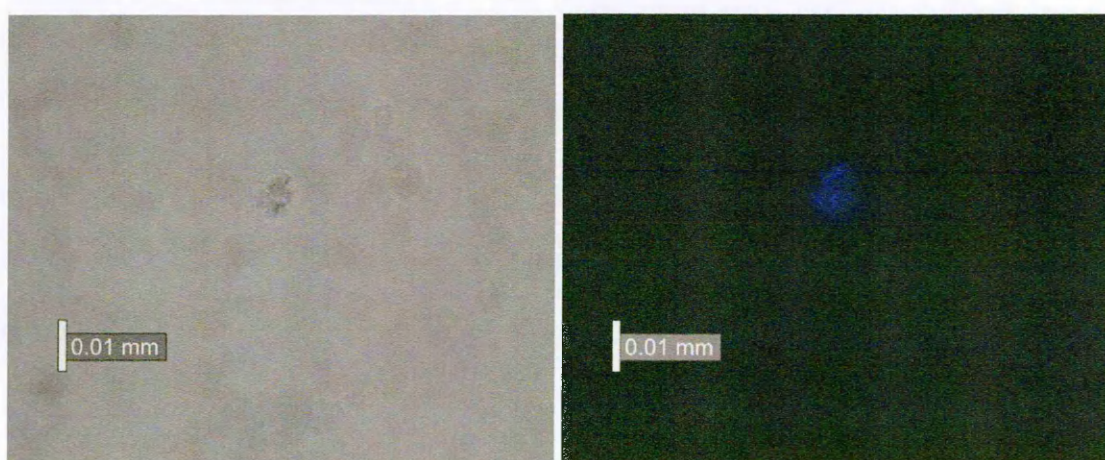
Figure 48 - CBIS 16 Isolate 3 (CBIS 16 I3) retrieved from the final dilution in MPN experiments from sediments at 571.1 m. Images are shown in white light (left) and stained with DAPI (right).

CBIS 19 Enrichment – 643.5 meters

Serial dilutions of CBIS 19E provided isolates in liquid culture. Organisms that were successfully grown in final dilutions were often difficult to transfer into fresh media. In most cases, these dilution isolates died off if transferred into fresh media, and in order to keep cultures alive, fresh media had to be titrated into the original culture. Only one isolate obtained from CBIS 19E showed successful growth on plates after isolation. This isolate (CBIS 19I2 below) was successfully grown only on soft-top agar plates.

The first isolate, CBIS 19I1, was obtained through serial dilution in liquid media from the original enrichment culture CBIS 19E, and was composed of thin rod-like cells (**Figure 49**). These cells often floated freely, and sometimes could be seen in small groups. Cells ranged from 5 μm to 15 μm in length. Elongated cells often began to take on a crescent shape. Attempts to grow CBIS 19I1 on solid and soft-top agar plates failed in all attempts.
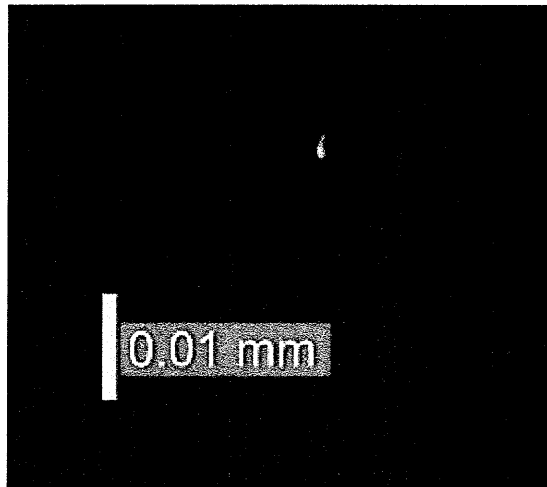
Figure 49 - A rod-shaped cell in isolate culture CBIS 19I1 stained with DAPI. The movement of free cells in liquid media made photographing without blurring difficult at the longer exposure times required for imaging during epifluorescence microscopy.

CIBS 19 Isolate 2 (CBIS 19I2) was obtained through serial dilution of CBIS 19E, and was the final culture remaining after three dilution steps. Cells were small, rod shaped, and seen primarily in clumps (**Figure 50**). Clumping made individual cells difficult to see and image. Attempts to grow CBIS 19I2 on solid media failed; however, growth was successful on soft-top agar plates, which contained a solid media base coated with a semi-

solid layer of media containing a reduced agar concentration. This colony (**Figure 51**) began growing at a single point near the surface of the semi-solid agar media, and then spread downward through the media to the interface between the solid and semi-solid media. From here, the colony continued to spread outward along the solid agar surface.
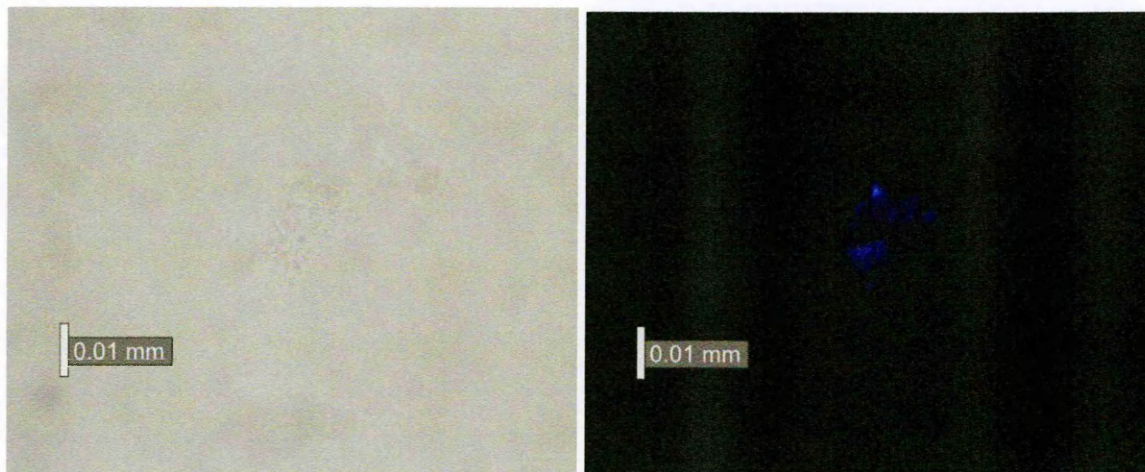
Figure 50 - CBIS 19I2

Figure 51 - CBIS 19 I2 grown on soft top agar plate.

A third dilution isolate (CBIS 19I3) from enrichment cultures appeared similar to CBIS 19I2. Cells were small and often seen in clumps (**Figure 52**). Individual cells were difficult

to distinguish. It also appeared that cells in clumps were surrounded and inundated with non-cellular material.
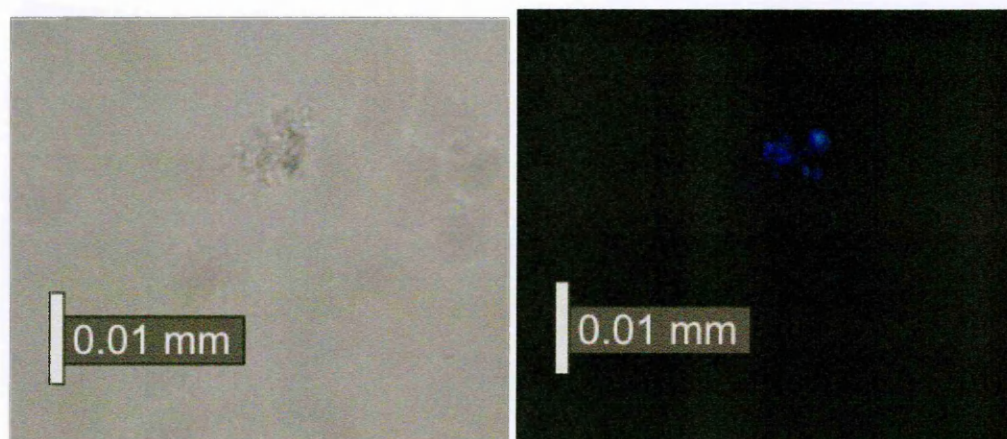
Figure 52 - CBIS 19I3 in white light (left) and stained with DAPI (right).

Because cells were often collected in masses of material that did not fluoresce when stained, it was thought that the cells themselves might be forming extracellular materials, such as polysaccharides (**Figure 53**). Even single cells in culture often had significant amounts of non-fluorescing material associated with them (**Figure 54**).
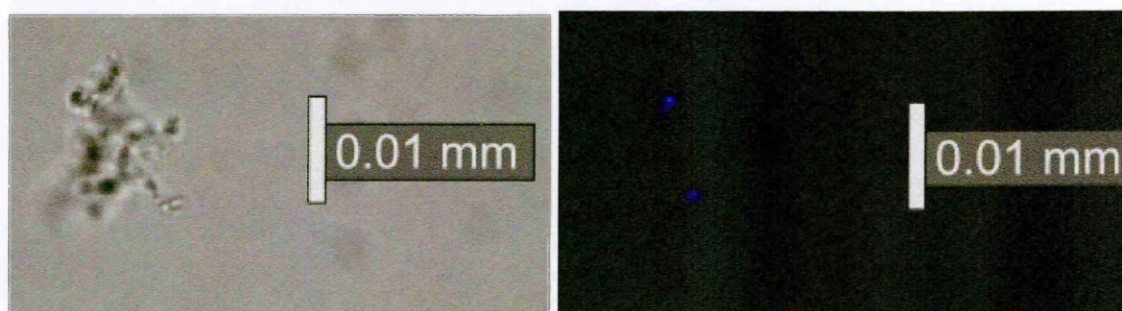
Figure 53 - CBIS 19I3 culture shown stained with DAPI (left) and under white light (right).
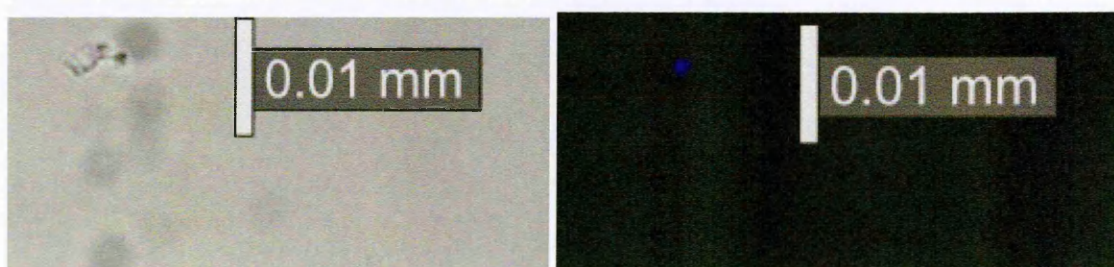
Figure 54 - Single cell of CBIS 19I3 shown stained with DAPI (right) and under white light (left). The cell (stained blue) is associated with material that does not fluoresce with DAPI staining.

A fourth isolate, CBIS 19I4, again showed no significant difference from other isolates obtained from CBIS 19E. Cells were thin, strand-like and often collected together in clumps (**Figure 55**). Single cells were not often viewable under the microscope.
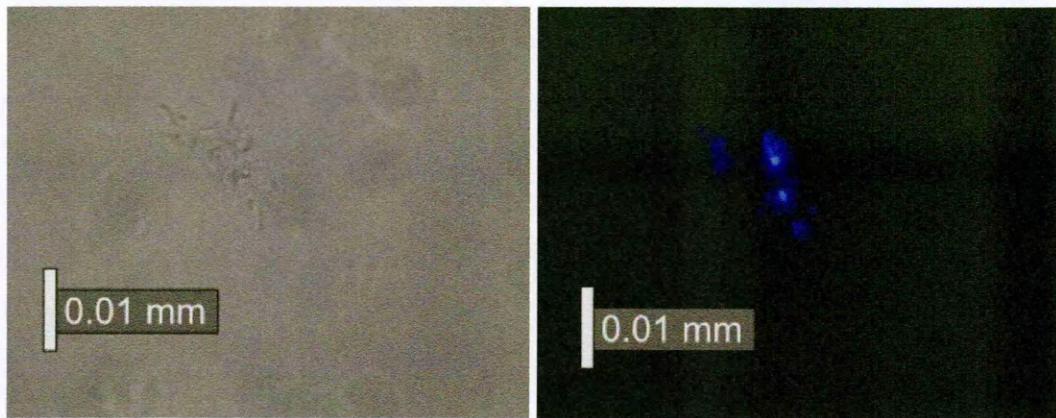
Figure 55 - Images of CBIS 19I4 photographed in white light (left) and stained with DAPI (right).

CBIS 28 Enrichment – 842.1 meters

One liquid dilution of CBIS 28E proved successful and was dubbed CBIS 28I1. Cultures were short-lived, and although growth could occur in the first week after inoculation, cells were undetectable after >20 days of incubation. No amplifiable DNA was obtained from any isolate at this depth.

CBIS 45 Enrichment - 1568.5 m

Dilution isolates obtained from CBIS 45E resembled the cells predominant in enrichment cultures. Cells were spherical and were seen floating as individuals, in pairs and often in clusters of >20 cells. When cells were plated onto solid plates after only two or three dilutions they would yield successful colonies. However, beyond three rounds of dilutions in liquid culture, colonies on solid media could no longer be obtained.

The first of the three isolates obtained, CBIS 45 I1, was obtained through direct serial dilution from CBIS 45E and is shown in **Figure 56** and **Figure 57.**
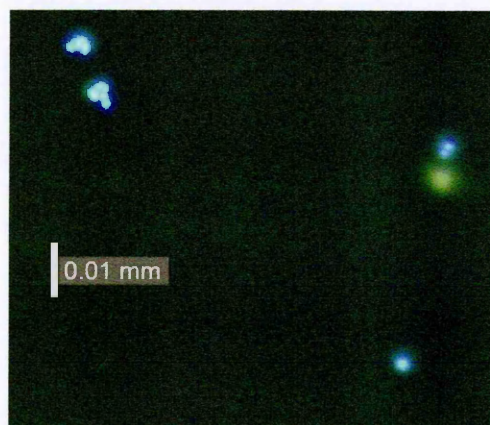
Figure 56 - CBIS 45I1 stained with DAPI in liquid culture.
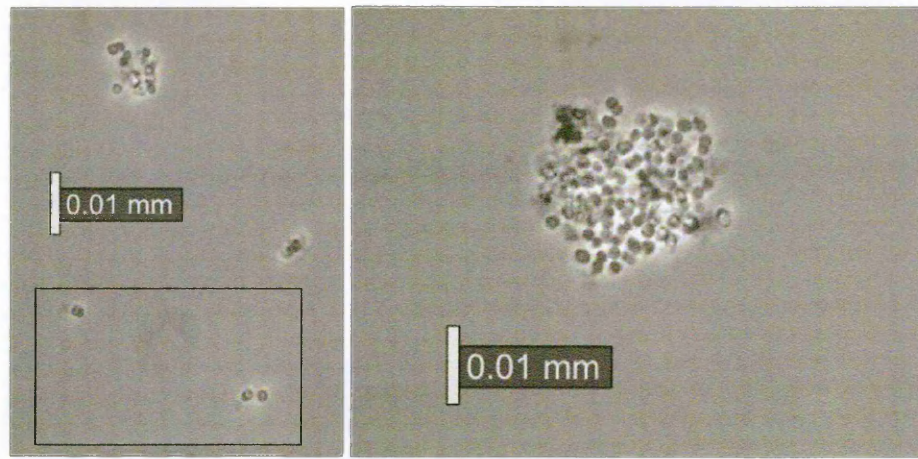
Figure 57 - CBIS 45 I1 seen with cells grouped together and as individuals.

CBIS 45 I2 and CBIS 45 I3 were collected from lanes one and two of MPN tests for sediments at depth 45 (1568.5 m). Cells in both isolates appeared to be the same as those seen in CBIS 45 I1 and in the enrichment cultures CBIS 45E (data not shown).

CBIS 45 I4 was collected from lane 3 of the MNP tests. Cells in this isolate appeared different than those seen in other 45 isolates. Cells were thin, elongated and formed small clumps in liquid culture (**Figure 58**). When removed from the MPN bottle and inoculated into fresh media, CBIS 45 I4 was able to continue growth in only the first set of inoculations. Transferring cultures from these inoculations to further bottles of fresh media proved unsuccessful. Additionally, CBIS 45 I4 was short-lived and cultures could not be maintained beyond 30 days. No amplifiable DNA was obtained from CBIS 45 I4.
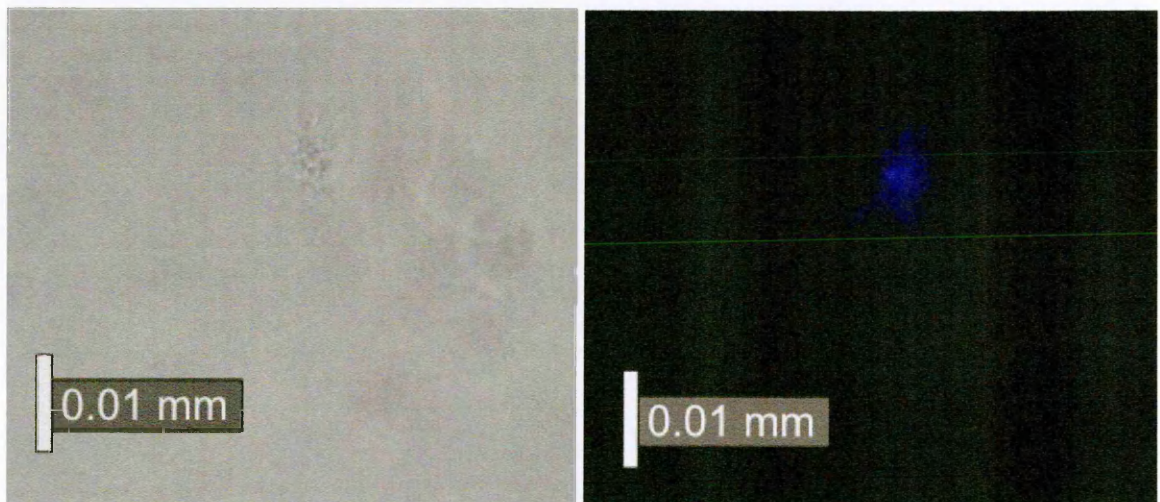
Figure 58 - CBIS 45 I4, obtained from MPN dilutions of CBIS sediments from 1568.5 m, shown in white light (left) and stained with DAPI (right).

CBIS 46 Enrichment - 1608.8 m

Enrichment cultures from a depth of 1608.8 m (CBIS 46E) were similar to CBIS 45E,

except for the fact that cells, while ovular and coccoid, were in general smaller and had

less of a tendency to clump together. Cells could sometimes be seen floating in pairs or

triplets, but it was rare to see clumps of >5 cells.

The same was true in dilution isolates obtained from CBIS 46E. The first dilution

isolate (CBIS 46I1) appeared almost identical to CBIS 46E. Cells were small, spherical

and free floating in liquid culture.
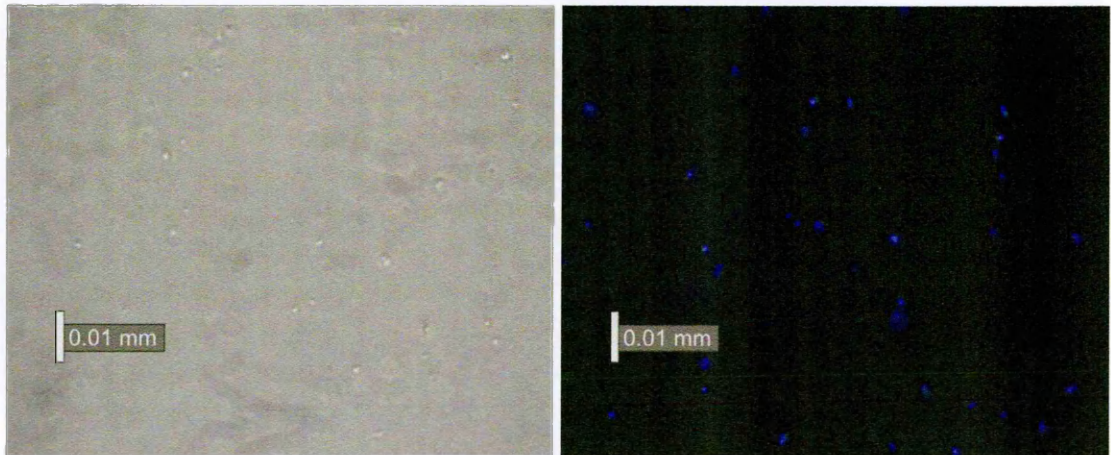
Figure 59 - CBIS 46 I1 in white light (left) and stained with DAPI (right).

CBIS 46I1 produced successful cultures when spread on solid agar plates (**Figure 60** and

Figure 61). Colonies were similar to those seen when the enrichment culture CBIS 46E

was spread on plates. Colonies began as a single point and spread outward during

incubation to reach a final size of ~5 mm in diameter.
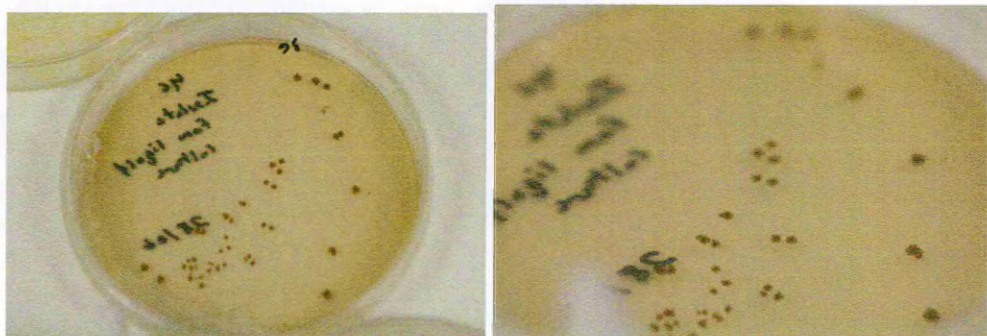
Figure 60 - Colonies of CBIS 46I1 when spread on solid plates of Het Med 1
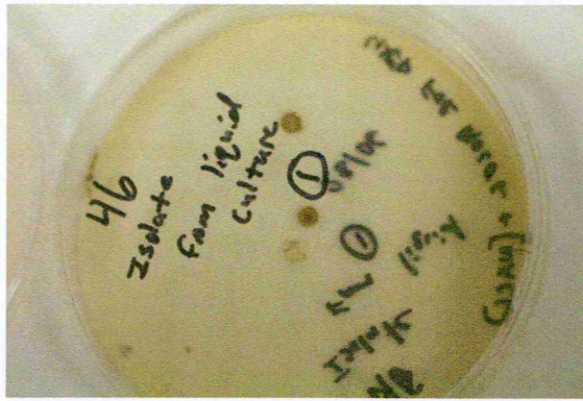
Figure 61 - Colonies of CBIS 46 I1 picked and re-spread on solid Het Med 1 agar plates.

When the anaerobic chamber failed and the internal atmosphere became oxic, plate

cultures of CBIS 46I1 were removed from plates and returned to liquid media. Cells

numbers failed to show growth, and only mat-like clusters could be seen under the

microscope, which were likely pieces of the original plate culture that was removed from

solid media (**Figure 62**). No free-floating cells could be found in the media.
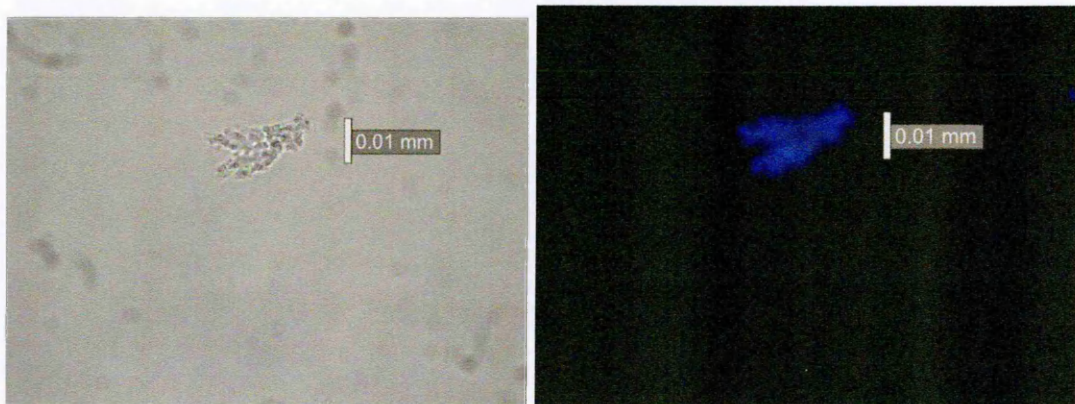
Figure 62 - Plate culture of CBIS 46I1 returned to liquid culture shown in white light (left) and stained with DAPI (right).

The second dilution isolate (CBIS 46I2) obtained from enrichment cultures appeared to be

identical to CBIS 46I1 in terms of morphology (**Figure 63**). Attempts to grow CBIS 46I2

on solid plates were not attempted due to failure of the anaerobic chamber.
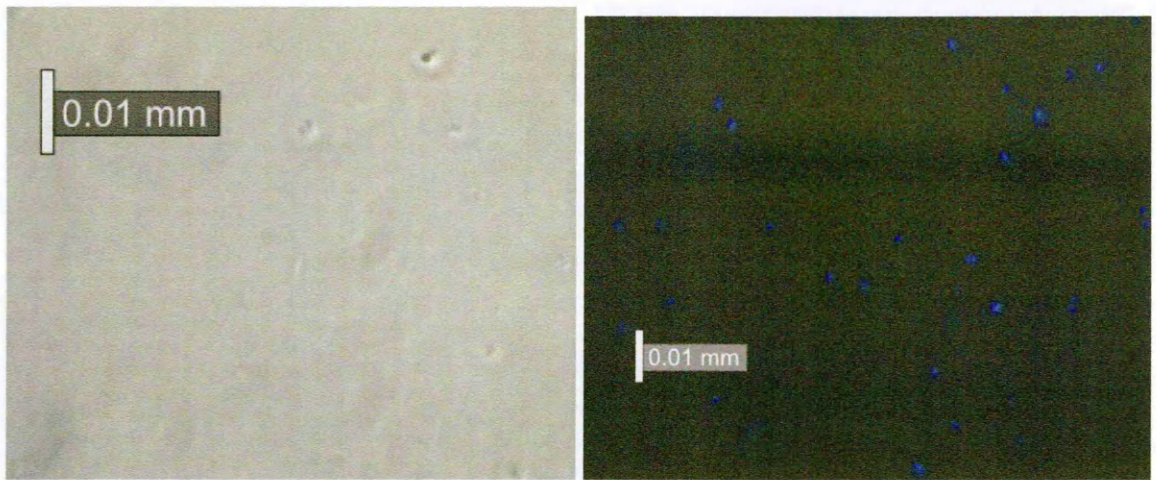
Figure 63 - CBIS 46I2 shown in white light (left) and stained with DAPI (right).

A third isolate, CBIS 46I3, was removed from 46MPN tests. CBIS 46I3 also appeared to be similar to CBIS 46I1 and CBIS 46I2. Cells were spherical and typically seen as individuals in liquid media. Occasionally, small groups of 2-5 cells could be seen. Staining with DAPI sometimes caused fluorescence only in the centre of cells, making cells under epifluorescence appear smaller than when viewed under white light (**Figure 64**).
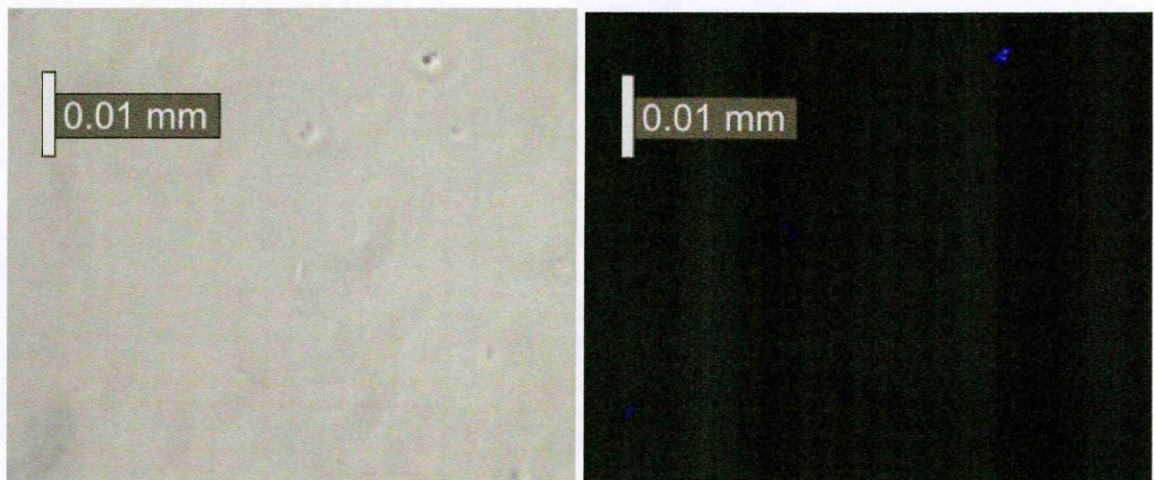
Figure 64 - CBIS 46 I3 shown in white light (left) and stained with DAPI (right).

CBIS 39-1 Fe USGS

Enrichment cultures obtained with the Fe USGS media for iron reducers were diluted across fresh liquid media. Growth occurred through eight separate inoculations. Cell types present in the final dilution appeared to be identical to those in the original enrichment cultures (**Figure 65**). These were single, rod-shaped cells floating freely in the liquid media, and dubbed CBIS 39-1 Isolate. Cells did not appear to associate directly with the iron precipitates present in the media.
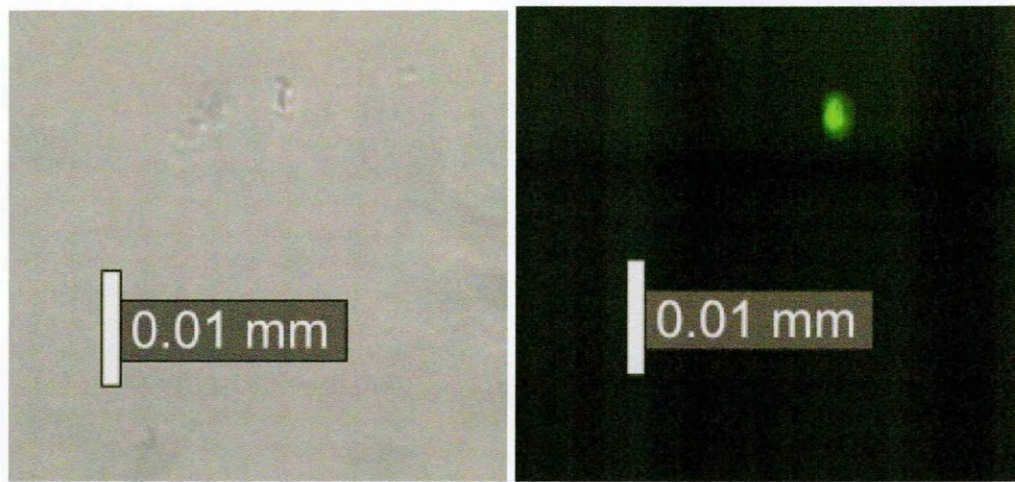
Figure 65 - CBIS 39-1 Isolate from serial dilutions in liquid media shown in white light (left) and stained with Syto 9 (right).

5.4.3 Isolates Obtained Through Plate Spreading

Not all enrichment cultures obtained with Het Med 1 could successfully be grown on solid media. For those cultures that did deliver colonies on agar plates, not all could be successfully transferred to fresh plates to produce growth. When colonies could be picked and spread onto fresh plates, growth typically occurred only on the first plate to which colonies were transferred. Further plating proved unsuccessful with the majority of cultures.

Additionally, failures with the anaerobic chamber meant that many cultures were lost due to oxygenation of the atmosphere inside the chamber. When this occurred, plate colonies were transferred to liquid culture. Only those that could be successfully re-inoculated into liquid to produce growing cultures were recovered.

CBIS 16E – 571.1 meters

When spread on agar plates, CBIS 16E produced a multitude of small colonies. After incubation, colonies did not increase in area and remained as pin-prick sized collections of cells. Colonies picked and spread on new plates did not grow in any attempts. When re-suspended in liquid and viewed under the microscope, plate colonies of CBIS 16E appeared to be composed of single cells, often grouped in chains. All individual colonies viewed under the microscope appeared similar in terms of the cell types present.

Figure 66 - CBIS 16E spread on sold Het Med 1 agar plates.

Because individual colonies of CBIS 16E could not be successfully spread on fresh plates, DNA was extracted from single colonies in an attempt to determine whether or not multiple sequences were present or if a single clean sequence could be used to identify the species types of organisms present. Plate colonies that could be re-cultured in liquid media were then designated CBIS 16 PI.
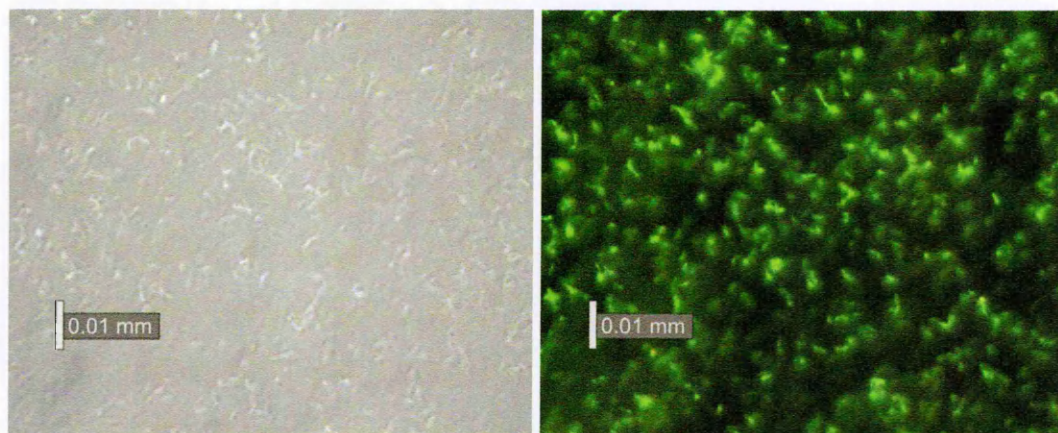
Figure 67 - CBIS 16 Plate Isolate (CBIS 16PI), obtained from enrichment cultures (CBIS 16E) from CBIS sediments at a depth of 571.1 m and shown in white light (left) and stained with Syto 9 (right).

CBIS 19E – 643.5 meters

Plate spreading of CBIS 19E produced a multitude of colonies on solid Het Med 1 agar plates. Colonies were picked and spread on fresh media plates. After one round of spreading, colonies were no longer able to be picked and transferred to fresh plates. Single colonies that were successfully grown in the first round of spreading were dubbed CBIS 19PI (**Figure 68**). CBIS 19PI was incubated in the anaerobic chamber, and when the anaerobic atmosphere failed, colonies were picked and transferred to liquid media in anaerobic culture bottles. Cultures were successfully re-grown in liquid media (**Figure 69**).
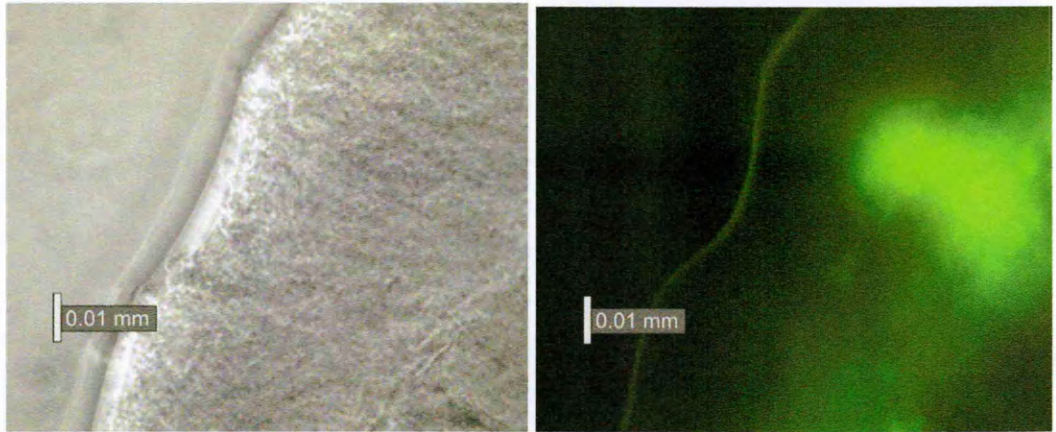
Figure 68 - CBIS 19 PI viewed at 1000x in white light (left) and stained with Syto 9 (right).

CBIS 19PI
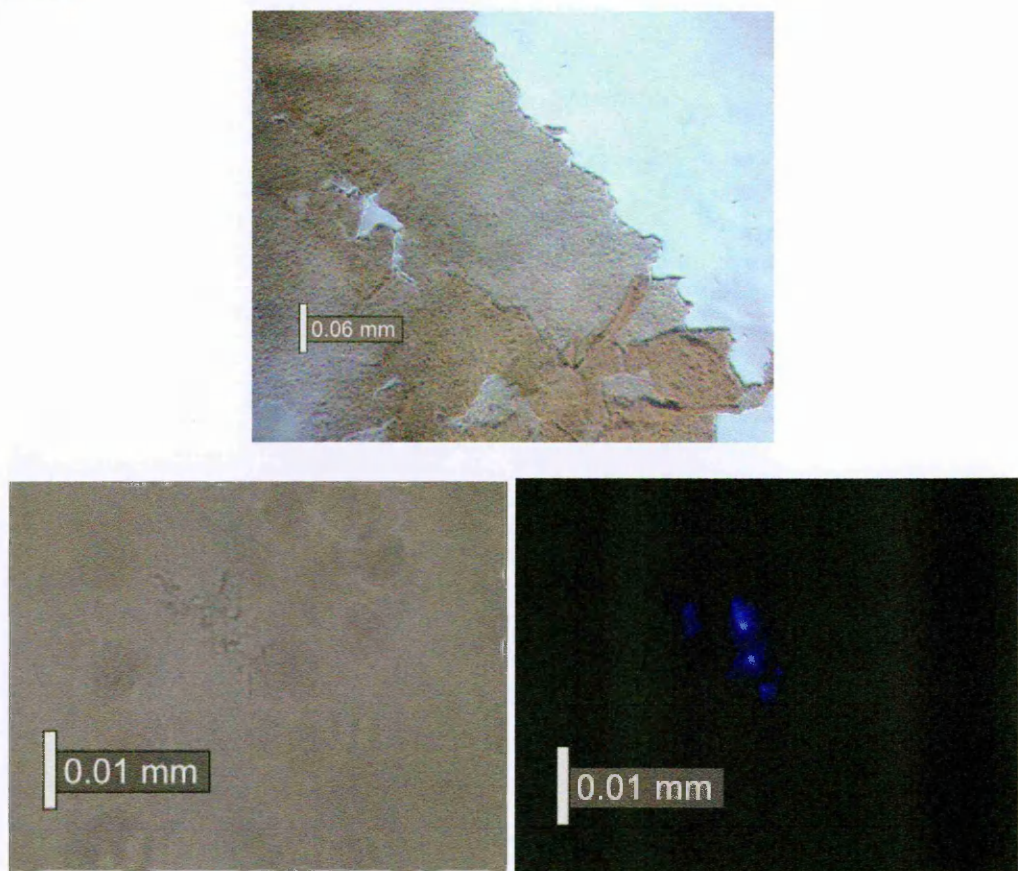
Figure 69 - CBIS 19 PI re-suspended in liquid and viewed under white light (left) and stained with DAPI under epifluorescence (right) at 1000x.

CBIS 28E – 842.1 meters

CBIS 28E could not be cultured on solid Het Med 1 agar plates. However, when liquid

CBIS 28E was spread on soft-top agar plates, successful colonies were formed (**Figure**

70). Cultures grew in the semi-solid agar media in columns. Within the first two weeks of

incubations, these columns extended down from the surface of the semi-solid media to the

interface between the semi-solid media and the solid media base. From this point on, colonies did not expand in size.
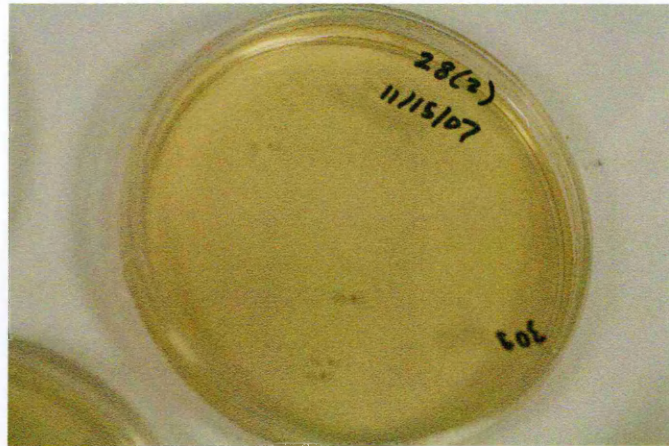
Figure 70 - CBIS 28 PI grown on soft-top agar plates.

When the anaerobic chamber failed, colonies were transferred to liquid Het Med 1. Removing colonies from semi-solid agar media was difficult, and complete extraction from the agar was often not possible. Re-growth of colonies in liquid media was rarely successful, but two culture bottles showed growth after inoculation. DNA was extracted from cultures re-suspended in liquid media and used for 16S rDNA sequencing.

CBIS 39-1 Fe Enrichment – 1463 m

Liquid enrichment cultures were obtained with Fe USGS media for iron reducers at a depth of 1463 m. Enrichment cultures produced thick, film-like colonies on plates (**Figure 71**). When spread onto fresh plates, colonies were successfully re-grown and dubbed CBIS 39-1 PI. These colonies proved to be the easiest to grow on solid plates, and survived over a series of spreading on >5 plates before failure of the anaerobic chamber meant that plate colonies had to be re-inoculated into liquid Fe USGS media.
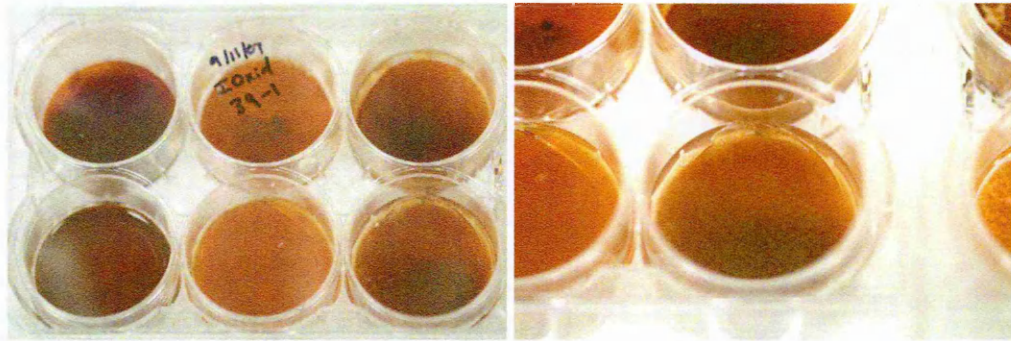
Figure 71 - Cultures of CBIS 39-1 Fe USGS spread across plates containing solid Fe USGS media.

In liquid culture, CBIS 39-1 PI appeared similar to cells in CBIS 39-1 enrichment cultures and CBIS 39-1 Isolate. Cells were small, rod-shaped individuals floating freely in liquid and appeared not to associate with iron precipitates in the media.

DNA extractions with all attempted methods failed with CBIS 39-1 I. One successful extraction was achieved with the enrichment culture CBIS 39-1 using the FastKit DNA extraction method. However, this extracted DNA produced unreadable sequences when PCR amplification was attempted with a multitude of primers (see below, **Table 28**).

CBIS 45E – 1568.5 metres

CBIS 45E successfully produced colonies on plates, all of which appeared to be composed of dense colonies of cells similar to those in original enrichment cultures of CBIS 45E. Individual colonies on the enrichment plates also appeared to be of similar composition. Colonies were transferred from the original plate and spread on consecutive plates to obtain isolates; however, colonies would only grow after the first transfer to fresh plates. These colonies were then removed and transferred to liquid media when the anaerobic chamber failed, and dubbed CBIS 45 PI. All attempts at DNA extraction from these samples failed.
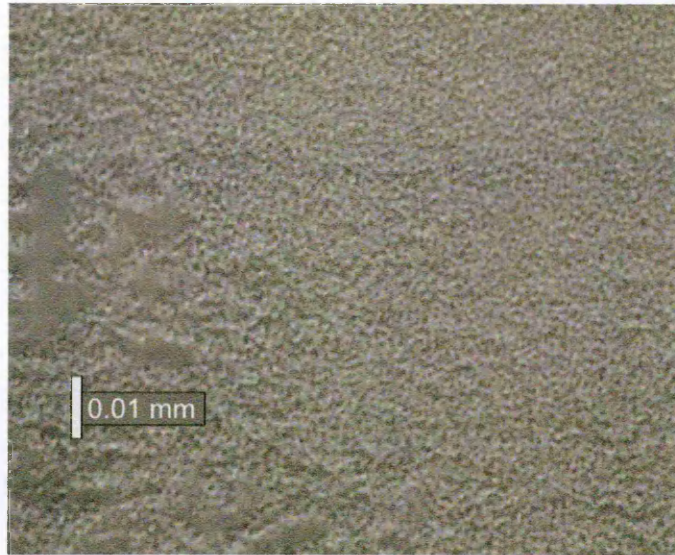
Figure 72 - Plate isolate (CBIS 45PI) obtained from spreading colonies on solid Het Med 1 agar plates.

CBIS 46E – 1608.8 meters

CBIS 46E successfully produced colonies on solid Het Med 1 agar plates. Colonies were

picked and re-spread over fresh plates to produce isolates. Spreading was successful over

two to three plates before colonies would no longer grow.
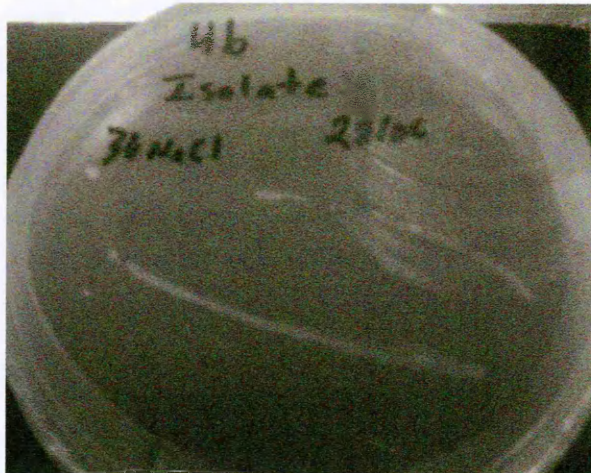
Figure 73 - CBIS 46I1 streaked on solid Het Med 1 agar plates.

On plates, colonies began to grow as a single point and expanded outward to ~5 mm in

diameter during incubation at 37°C. Colonies on the final plates were collect and placed in

liquid media when the anaerobic chamber failed. One isolate was re-grown in liquid media

and dubbed CBIS 46PI. Very few cells were present in the culture of CBIS 46 PI that was

recovered in liquid media, and DNA extracted by all attempted methods provided no

amplifiable product.

5.4.4 Extractions from Enrichment Cultures

DNA extraction, PCR amplification and 16S rDNA sequencing was performed directly on

three enrichment cultures in liquid media: CBIS 16E (571.1 m), CBIS 19E (643.5 m) and

CBIS 45E (1568.5 m). This was done because enrichment cultures all appeared to be

composed of single cell types under the microscope, and it was thought that the

enrichments could actually be single-species cultures. Amplifiable DNA and clean

sequences were successfully obtained from all enrichment samples. Sequences were

compared to known sequences in the NCBI nucleotide Blast database

(http://blast.ncbi.nlm.nih.gov/Blast.cgi). The closest matching sequences for each culture

are shown in **Table 26**.

Table 26 - Closest matching sequences for sequences obtained directly from enrichment cultures. G+C estimates are based on 16S rDNA sequences, the lengths of which are listed in base pairs (bp). Matches marked (*) indicate species that have been suggested to be identical. N/A indicates species whose origins were not indicated in the NCBI Blast database. NCBI accession numbers are listed following the names of closest matches.

CBIC Sample	Depth (m)	G+C est.	Closest Matches	Coverage	Max Ident.	Cultured From
CBIS 16E	571.1	55.5% 1462 bp	*Jonesia* sp. YNUCC0043 (DQ112344.1)	98%	95%	N/A
			Jonesia qinghaiensis (AJ626896.1)	98%	95%	Soda lake mud (Schumman *et al.* 2004)
			Sanguibacter sp. (AB126692.1)	97%	94%	Sagara oil reservoir
CBIS 19E	643.5	56.2% 1428 bp	*Bacillus* sp. PeC11 (AM177061.1)	99%	99%	Alkaline guts of soil feeding insects (study on Fe(III) reduction by bacillus strains)
			Bacillus sp. HZBN43 (EF625229.1)	99%	99%	China Sea
			Bacillus sp. BMP-1 (DQ371431.1)	100%	99%	Waste-water treatment (Perez-Ibarra *et al.* 2007)
			Bacillus boroniphilus EU620409	99%	99%	Palk Bay (Thondi) sediments
CBIS 45E	1568.5	53.5% 1511 bp	*Trichococcus collinsii* (AJ306612.1)	100%	99%	N/A
			L.pasteurii (X87150.1)	100%	99%	(Janssen *et al.* 1995)
			Nostocoida limicola (AF255736.1)	99%	99%	Biowaste reactor
			Carnobacteriaceae bacterium (AB298778.2)	98%	99%	Methanogenic fermenter of cattle waste
			Trichococcus patagoniensis (AF394926.1)	97%	99%	Penguin guano in Patagonia (Pikuta *et al.* 2006)

5.5 Freeze-Thaw Lysis and FastKit DNA Extraction

The Freeze-Thaw (FT) lysis method was performed in two iterations on a total of 39

culture samples. The FT method was tested because it can be performed quickly on a large

number of samples, and without the use of expensive commercial kits. When analyzing

PCR products on the 0.8% agarose gels, none of the extractions provided successful

amplification product (i.e. **Figure 74**). PCR on freeze-thaw lysis samples also showed no

detectable DNA when product was analyzed on the NanoDrop (data not shown).
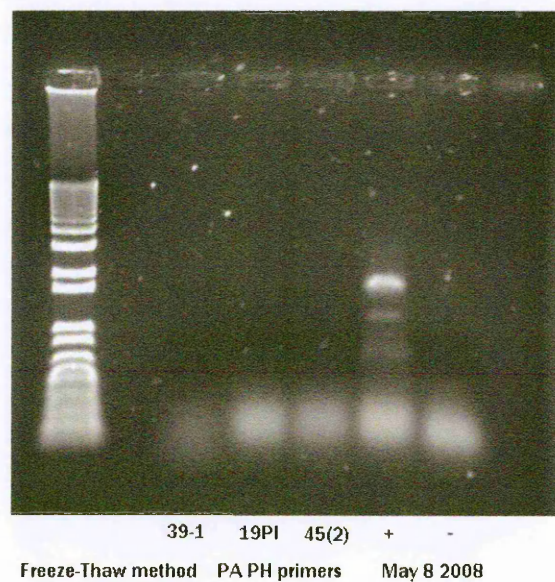
Figure 74 – An example of a 0.8% agarose gel of FT extractions from three CBIS cultures. Only the positive control (+) showed amplified DNA product.

When the FT method failed to produce useable DNA extractions, DNA was acquired from

a selection of samples using the FastKit DNA Extraction Kit. Not all 39 FT samples were

used for DNA extraction due to the prohibitory cost of the commercial kit. **Table 27** lists

Table 27 - FastKit DNA extraction product from a selection of samples where freeze-thaw extraction failed to yield amplifiable DNA.

Culture Labels	Depth	mL DNA extracted
CBIS 16 I1	571.6	48.5 µg/mL
CBIS 16 I1	571.6	138.2 µg/mL
CBIS 19 PI	652.2	47.8 µg/mL
CBIS 28 ST	842.1	49.7 µg/mL
CBIS 28 E	842.1	36.7 µg/mL
CBIS 39-1	1463	129.9 µg/mL
CBIS 45I1	1587.3	30.5 µg/mL
CBIS 45 IOE	1587.3	114.8 µg/mL
CBIS 46 PI	1614.9	46.9 µg/mL

the cultures from which DNA was extracted and the estimated amount of DNA successfully extracted when products were examined on the NanoDrop.

Successful extractions were then brought to the USGS in Reston, VA, and PCR was performed using the primers listed in **Table 28**. PCR produced successful products from eight of the extractions. The largest segments were obtained with the primers 46f/1387r. However, when these large segments were sequenced, they produced many peaks that were difficult to interpret, meaning that distinct base pairs could not be determined for significant portions of the sequence. Shorter sequences were then produced using 46f/1100r and 46f/519r in an attempt to obtain PCR product that could be sequenced with higher confidence.

Table 28 - PCR product from FastKit extracted DNA performed with a selection of primers. N/A indicates samples where specific primers were not used for PCR amplification. (+) indicates successful PCR amplification product. ("+") Indicates PCR products that were successful but showed only a weak band when run on the gel. (-) indicates unsuccessful PCR amplification. Products highlighted in grey are those that were ultimately used for sequencing. Hatched boxes indicated PCR products that were unreadable when sequencing was attempted.

Culture Labels	Depth (m)	46f / 1387r	46f / 519r	46f / 1100r	Geobacter	Sbr
CBSI 16 I1	571.6	+	"+"	+	N/A	-
CBIS 16 I1		+	-	-	N/A	-
CBIS 19 PI	652.2	+	-	-	N/A	N/A
CBIS 19 PI (1/10 dilution)		-	+	+	N/A	N/A
CBIS 28 ST	824.1	+	+	+	N/A	N/A
CBIS 28E		+	+	+	-	-
CBIS 39-1 Enrichment	1463	+	+	+	N/A	N/A
CBIS 45 E	1587.3	+	+	+	N/A	N/A
CBIS 45 IO E		+	+	+	N/A	N/A
CBIS 46 PI	1614.9	+	-	-	N/A	-

Sequencing was performed on PCR products obtained from cultures in **Table 28**. Successful PCR products were purified using the Wizard PCR purification kit (Promega, Madison, WI). When PCR product could not be obtained for large segments of the 16S rDNA sequence (i.e. using primers 1387r or 1100r), smaller internal fragments of the DNA were attempted using 519r. In some cases, large segments of DNA that were obtained contained a high number of errors when sequenced, or in other cases showed no sequence at all. Ultimately, all samples were sequenced with the selection of Eubacterial primers in

Table 28. After analyzing the obtained sequences, the cleanest sequence for each culture was used to identify closest matching known organisms in the NCBI Blast database.

DNA was extracted from two replicate cultures of CBIS 16I1. Only one of these cultures returned amplifiable DNA. DNA from CBIS 19PI was also unusable; however, a 1:10 dilution of the DNA extraction product from CBIS 19PI did return PCR product. Only a faint band was seen on the gel for CBIS 46PI using the primers 46f/1387r, however PCR product showed no sequence when analyzed. No additional product could be obtained from CBIS 46PI. When sequence from CBIS 39-1 using 46f/1387r was run on the analyzer, the sequence also came up blank. However, additional product from CBIS 39-1 E was obtained using primers 46f/1100r and 46f/519r. DNA fragments with these primers returned sequences that contained many errors, and large segments of the sequence could not be read. When run in the NCBI database, the sequences showed only low similarity to uncultured and unidentified bacterial clones. Ultimately, due to the amount of errors present in sequences from CBIS 39-1, they were deemed unreadable and discarded.

Sequencing of the enrichment cultures CBIS 28E and 45 IO E was performed even though these cultures were original enrichment cultures obtained by inoculating media with CBIS core sediments. As with other enrichment cultures that were directly sequenced (above), these cultures appeared to be single cell types under the microscope and we were interested in seeing if clean sequence could be obtained. Sequences obtained from both of these samples contained numerous errors and base pairs that could not be interpreted. When these sequences were run in the NCBI database, they returned only low matches with uncultured and unidentified bacterial clones. These samples were then dubbed unreadable.

The cleanest sequences (highlighted in **Table 28**) were analyzed for closest matching organisms in the NCBI database by use of the NCBI nucleotide Blast. It should be noted that PCR product from many of these cultures returned only small fragments of the 16S rDNA sequence, and in many cases sequences had to be trimmed to remove

uninterpretable base pairs from the beginning and ends of sequences. Such short segments

do not necessarily provide a robust comparison with known sequences, and closest

matching organisms in the NCBI Blast database can only provide a general guide to the

identity of cultures.

CBIS 16 I1 (431 bp) 14% of sequence unreadable	Depth (m)	G+C Content	Closest Matches	Query Cvrg (%)	Max Iden (%)	Origin of Sequence in NCBI database
	571.1	50.1%	Uncultured bacterium clone (AB441513.1)	56	82	Microbial community in composting
			Uncultured bacterium clone (EU469634.1)	56	82	Faecal microbiota of mammals (Ley et al. 2008)
			Pseudomonas sp. D-14-25-5 (AB190114.1)	56	81	Microbial community in compost processing under cold climate

CBIS 19 PI (414 bp)	Depth (m)	G+C Content	Closest Matches	Query Cvrg (%)	Max Iden (%)	Origin of Sequence in NCBI database
	652.2	55.2%	Uncultured bacterium clone (EU369170.1)	98	98	Bacterial diversity in oyster shells
			Bacillus sp. K38T (AM983525.1)	98	98	Mediterranean anthropogenic soils
			Bacillus sp. SK50 (EU417669.1)	98	98	prokaryotic diversity of a landfill

CBIS 28ST (519 bp) Removed final 74 bp	Depth (m)	G+C Content	Closest Matches	Query Cvrg (%)	Max Iden (%)	Origin of Sequence in NCBI database
	842.1	53.2%	Uncultured bacterium clone (EU789856.1)	100	100	Dominant microbial population in Lascaux cave, France.
			Pseudomonas sp. 01WB04.4-39 (FM161591.1)	100	100	Cultureable aerobic bacteria from upstream of a karst water rivulet
			Pseudomonas sp. 01WB04.4-26 (FM161584.1)	100	100	Cultureable aerobic bacteria from upstream of a karst water rivulet

Additional PCR amplification was attempted using primers specific to iron reducing

bacteria (Geobacter) and sulfate reducing bacteria (Sbr) in an attempt to identify whether

or not organisms in cultures could belong to these metabolic groups (**Table 28**). CBIS 39-1

and CBIS 39-1I were cultures obtained with media specific to iron reducers (Fe USGS

Media), and PCR amplification with Geobacter primers would determine if sequences for

CBIS 39-1E showed similarity to sequences of known iron reducers. All other cultures

were obtained with heterotrophic media (Het Med 1); however, chemical data at depth in

the core indicated fluctuations in sulfate and iron levels at some of the depths from where

organisms were obtained (see **Chapter 4**). Additionally, the colour change seen in media

supporting cultures from a depth of 1614.9 m indicated the potential presence of sulfate

reduction (See **Chapter 4**). Because of this, primers specific to known sequences for

sulfate reducers (Sbr) were also used on samples in an attempt to identify the presence of

sulfate reducing microbes. Ultimately, no sequences were obtained from these tests (**Table**

28).

5.5.1 Direct PCR on Plate Colonies

The Direct PCR from Plate Colonies method was performed on CBIS plate cultures CBIS

16, CBIS 19 and CBIS 45 at the Open University. Attempts at amplifying DNA directly

from cell cultures failed in all attempts.

Plate colonies of 16, 19, 45 and 46 were then shipped to the USGS in Reston, VA,

for complete extraction and amplification. DNA was extracted and PCR was performed

using primers 63f and 1387r. Only one sequence was obtained using these primers (**Table**

29). For samples where these primers failed to produce sequences, additional cycles were

performed using archaeal primers (**Table 21**). These additional cycles also failed to

produce PCR product.

Table 29 – Results of PCR amplification from DNA extractions
performed directly on culture plate colonies on solid Het Med 1.

Culture Identifier	Depth (m)	Primers	
		63f/1387r	Archaeal f/r
CBIS 16PI	571.1	+	N/A
CBIS 19PI	652.2	-	-
CBIS 45PI	1587.3	-	-
CBIS 46 PI	1608.8	-	-

Successful PCR product from CBIS 16PI was sequenced on the ABI310 genetic analyzer

and the sequence was edited and assembled using Autoassembler. The sequence was

compared to known sequences in the NCBI Blast database. The closest matching

sequences are shown in **Table 30**.

Table 30 - Closest matching sequences in the NCBI blast database for CBIS 16PI. Estimated G+C content is also provided based on the 16S rDNA sequence of 1342 base pairs. Accession numbers from the NCBI database are listed in parenthesis following the identified closest matching sequences. Information on the origin of closest matching sequences or a published reference is provided.

CBIC Sample	Depth (m)	G+C est.	Closest Matches	Coverage	Max Ident.	Cultured From
CBIS 16 PI	571.1	56.3% 1342 bp	*J.denitrificans* (X83811.1)	100%	99%	(Rainey *et al.* 1995)
			Jonesia sp. TUT1015 (AB098583.1)	100%	99%	Compost reactor (Hiraishi *et al.* 2003)
			Sanguibacter sp. (AB126692.1)	100%	97%	Methanogenic fermenter of cattle waste
			Myceligenerans sp. XJ 11063 (EU910872.1)	99%	92%	Halophilic communities from Hami Lake

5.5.2 Optimal Growth Tests

Optimal growth tests were performed on three selected isolates that proved to be the easiest to culture across multiple inoculations. These three cultures included CBIS 16 I2, CBIS 19 I1 and CBIS 46 I1. Cultures of these isolates were incubated in order to produce as high a concentration of cells as possible before performing growth tests. It should be noted, that although these isolates were the easiest to culture, it was often difficult to achieve successful growth when cells were inoculated into fresh media even at high cell concentrations. This meant that numerous replicate tests were required in order to ensure that a failure of cell growth was due only to variations in the media conditions (i.e. pH, NaCl concentration, etc.) and not simply a failed inoculation.

5.5.3 Optimal Temperature

Optimal temperature experiments were performed with CBIS 16 I2, CBIS 19 I1 and CBIS 46 I1. Three attempts to perform optimal temperature experiments at each depth were made. Optimal temperature experiments failed in all attempts. Growth of cultures was erratic and even samples at the normal incubation temperature of 37°C sometimes failed to show significant growth. Because of this, optimal temperature experiments had to be abandoned.

5.5.4 CBIS 16I2, Het Med 1

Optimal NaCl

The behaviour of CBIS 16I2 in liquid culture made determining optimal growth for NaCl concentration difficult. Cells would grow singly or in small 'clumps' at low NaCl, but would then clump together in large 'masses' as NaCl concentration increased. This phenomenon has been observed before in cultures of non-coccoid haloarchaea, where increasing salt concentration causes morphological changes in cells (Fendrihan *et al.* 2006). Because of this, even when stained with DAPI, cell numbers were difficult to determine via microscopy. Additionally, the use of resazurin in culture medium and the relatively low density of cultures prevented the use of photometry to monitor growth.

An attempt was made to estimate the number of cells in 'clumps' and 'masses', and the total number of 'clumps' and 'masses' was then counted to yield a final rough estimate of cell numbers. Because each culture was counted by this method, a general comparison can be drawn between the experimental cultures. However, the estimate for total cell numbers obtained by this method should not be used outside of this context.

After incubation of cultures at 37°C for 20 days, cell cultures survived in concentrations of NaCL ranging from 0 g/L to 35 g/L. Cells were undetectable in [NaCl] 50 g/L and above after 13 days. Cells were undetectable in [NaCl] 45 g/L after 20 days. Highest cell numbers were achieved at around 35 g/L NaCl. The estimated concentration of NaCl at a depth of 571.1 m is in the range of 36.9 g/L.

After 13 days of incubations, cultures grown at NaCl concentrations in the range of 15 g/L to 45 g/L became difficult to count because clumping made it difficult to distinguish individual cells.
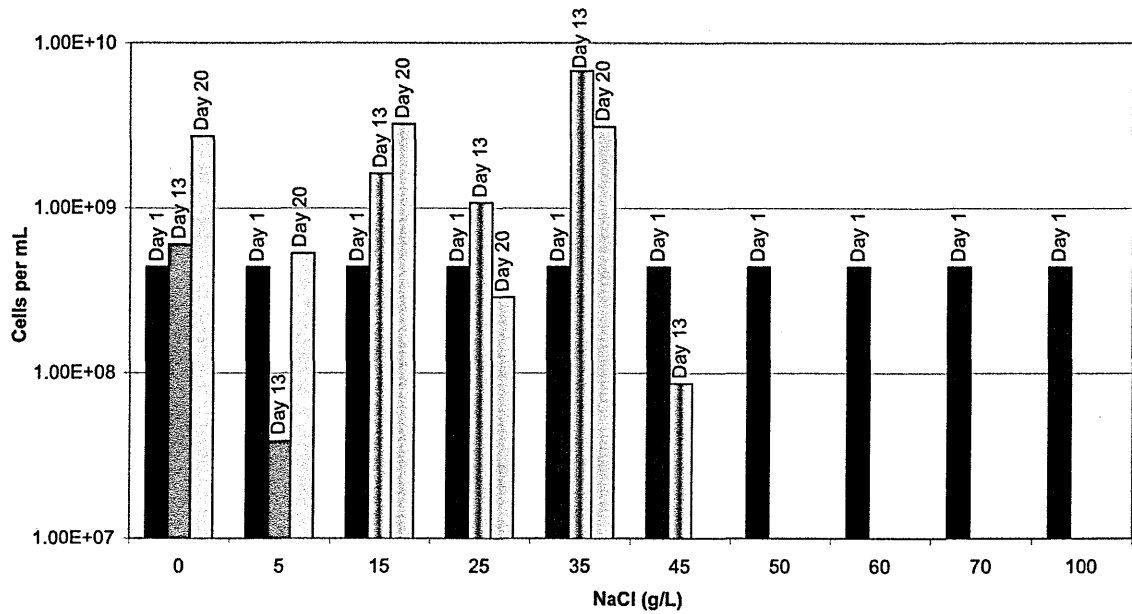
Figure 75 - Growth tests for CBIS 16 I2 at varying concentrations of NaCl. Thin bars indicate cultures where cells became clumped into large masses and mats, making it difficult to distinguish individual cells during counts.

Optimal pH

As with the determination of optimal NaCl concentration, behaviour of CBIS 16I2 in liquid culture made determining optimal pH for growth difficult. The same complications with cells growing as singly at the fringes of optimal pH, and then grouping together in large 'masses' at more optimal pH were observed. The counting difficulties and inability to use photometry due to resazurin present in the growth media made cell numbers difficult to estimate. The previous technique for counting 'clumps' and 'masses' rather than individual cells was employed to yield a basic understanding of how cell numbers were related between each of the cultures within the optimal pH experiments.

CBIS 16I2 was able to grow at pH values in the range of 6.0 to 9.0 after incubation at 39°C for 61 days (**Figure 76**). Cultures in solutions of pH 3.0 and pH 12.0 were undetectable at completion of the experiment.
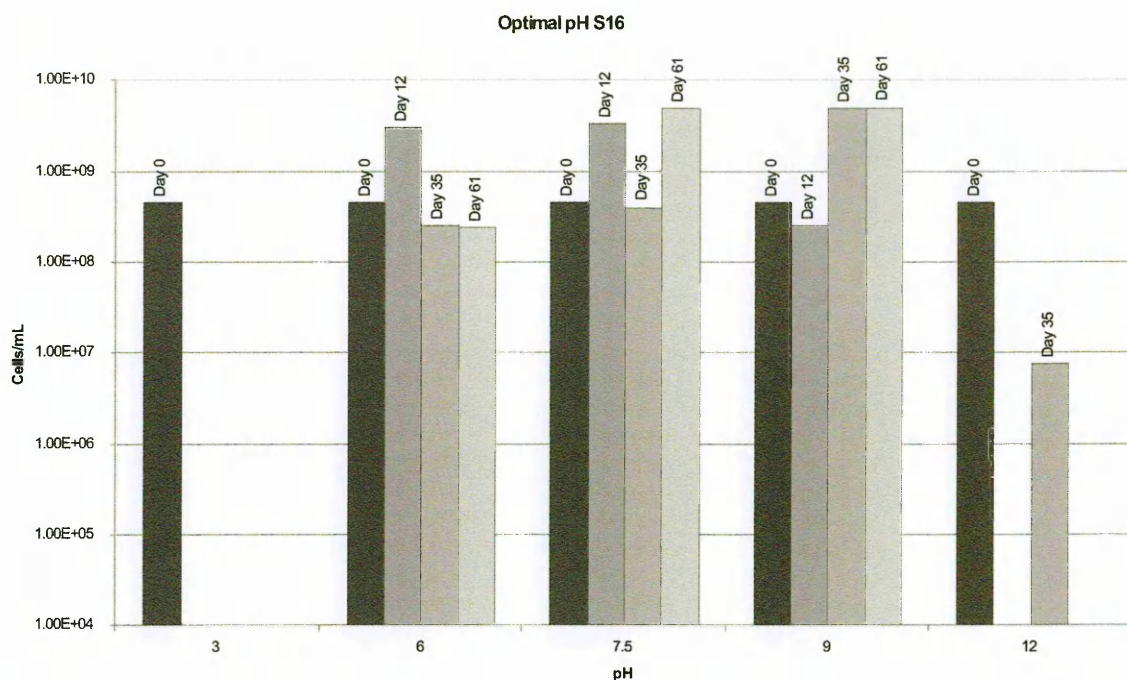
Figure 76 - Growth of CBIS 16I2 in pH ranging from 3.0 to 12.0. The starting concentration of cells in each culture is indicated by Day 0.

Carbon Source Utilization

The carbon source utilization profile based on results of the Biolog-AN Microplate is

shown in **Table 31** and **Figure 77**.

Water	N-Acetyl-D-Galactosamine	N-Acetyl-D-Glucosamine	N-Acetyl-β-D-Mannosamine	Adonitol	Amygdalin	D-Arabitol	Arbutin	D-Cellobiose	α-Cyclodextrin	β-Cyclodextrin	Dextrin
Dulcitol	i-Erythritol	D-Fructose	L-Fucose	D-Galactose	D-Galacturonic Acid	Gentiobiose	D-Gluconic Acid	D-Glucosaminic Acid	α-D-Glucose	α-D-Glucose-1-Phosphate	D-Glucose-6-Phosphate
Glycerol	D,L-α-Glycerol Phosphate	m-Inositol	α-D-Lactose	Lactulose	Maltose	Maltotriose	D-Mannitol	D-Mannose	D-Melezitose	D-Melibiose	3-Methyl-DGlucose
α-Methyl-D-Galactoside	β-Methyl-D-Galactoside	α-Methyl-D-Glucoside	β-Methyl-D-Glucoside	Palatinose	D-Raffinose	L-Rhamnose	Salicin	D-Sorbitol	Stachyose	Sucrose	D-Trehalose
Turanose	Acetic Acid	Formic Acid	Fumaric Acid	Glyoxylic Acid	α-Hydroxybutyric Acid	β-Hydroxybutyric Acid	Itaconic Acid	α-Ketobutyric Acid	α-Ketovaleric Acid	D,L-Lactic Acid	L-Lactic Acid
D-Lactic Acid Methyl Ester	D-Malic Acid	L-Malic Acid	Propionic Acid	Pyruvic Acid	Pyruvic Acid Methyl Ester	D-Saccharic Acid	Succinamic Acid	Succinic Acid	Succinic Acid Mono-Methyl Ester	m-Tartaric Acid	Urocanic Acid
Alaninamide	L-Alanine	L-Alanyl-LGlutamine	L-Alanyl-L-Histidine	L-Alanyl-L-Threonine	L-Asparagine	L-Glutamic Acid	L-Glutamine	Glycyl-L-Aspartic Acid	Glycyl-L-Glutamine	Glycyl-L-Methionine	Glycyl-LProline
L-Methionine	L-Phenylalanine	L-Serine	L-Threonine	L-Valine	L-Valine plus L-Aspartic Acid	2'-Deoxy Adenosine	Inosine	Thymidine	Uridine	Thymidine-5'-Mono-phosphate	Uridine-5'-Mono-phosphate

Figure 77: Microplate results for CBIS 16I2. Highlighted squares indicate wells where successful colour change occurred.

Only four of the 96 wells in the microplate showed a colour change indicating growth. A strong colour change was seen with D-Fructose, L-Fructose, Palatinose and L-Rhamnose.

Table 31 - Carbon source utilization by CBIS 16I2. Carbon sources marked "w" indicate only a weak colour change in the Biolog plate well.

Carbon Source	CBIS 46
D-Fructose	+
L-Fucose	+
Palatinose	+
L-Rhamnose	+

5.5.5 CBIS 19I1, Het Med 1

Optimal NaCl

Two replicate tests of optimal NaCl concentration were successful with CBIS 19I1. The estimated cell concentration in starting cultures for the first experiment was 2.29×10^8 cells/mL, and 1.52×10^8 cells/mL for the second experiment. Growth was monitored at NaCl concentrations of 0 g/L, 5 g/L, 15 g/L, 25 g/L, 35 g/L, 45 g/L, 55 g/L, 65 g/L, 75 g/L, 100 g/L.

Experiment 1 (**Figure 78**) showed stable cell cultures at all concentrations except for 55 g/L. At this concentration, cells were detectable up to 14 days of incubation, but could not be identified in solution after 21 days. In initial increase in cell numbers was seen only at a concentration of 35 g/L, close to the estimated concentration native to CBIS sediments at depths from which CBIS 19I1 was obtained (652.2 m). All other concentrations showed an initial decline in cell numbers.
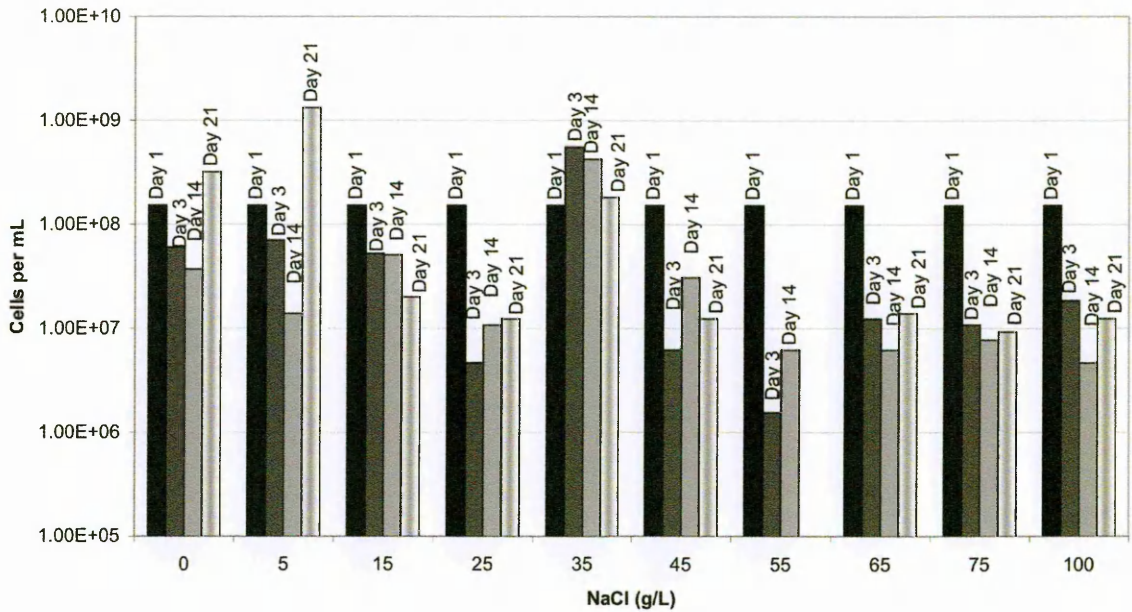
Figure 78 - Optimal NaCl experiment 1 for CBIS 19I1, Experiment 1. Thin bars indicate cultures where cells became clumped into large masses and mats, making it difficult to distinguish individual cells during counts.

Experiment 2 (**Figure 79**) showed growth at all concentrations tested. Unlike experiment 1, an initial decline in cell numbers was seen at all concentrations. For concentrations of 0 g/L and 100 g/L cell numbers decreased to levels that were undetectable under the microscope after 13 days and 3 days respectively. By 30 days of incubation, cell numbers in at these concentrations recovered to detectable levels. All other concentrations showed an initial decline in cell numbers. Cell numbers continued to decline across the incubation period for 15 g/L and 25 g/L. At other concentrations, cell numbers fluctuated around 1×10^7 cells/mL, with the lowest cell numbers seen at 45 g/L ($\sim 5 \times 10^6$ g/L). In all, relatively stable cultures were seen at all concentrations.
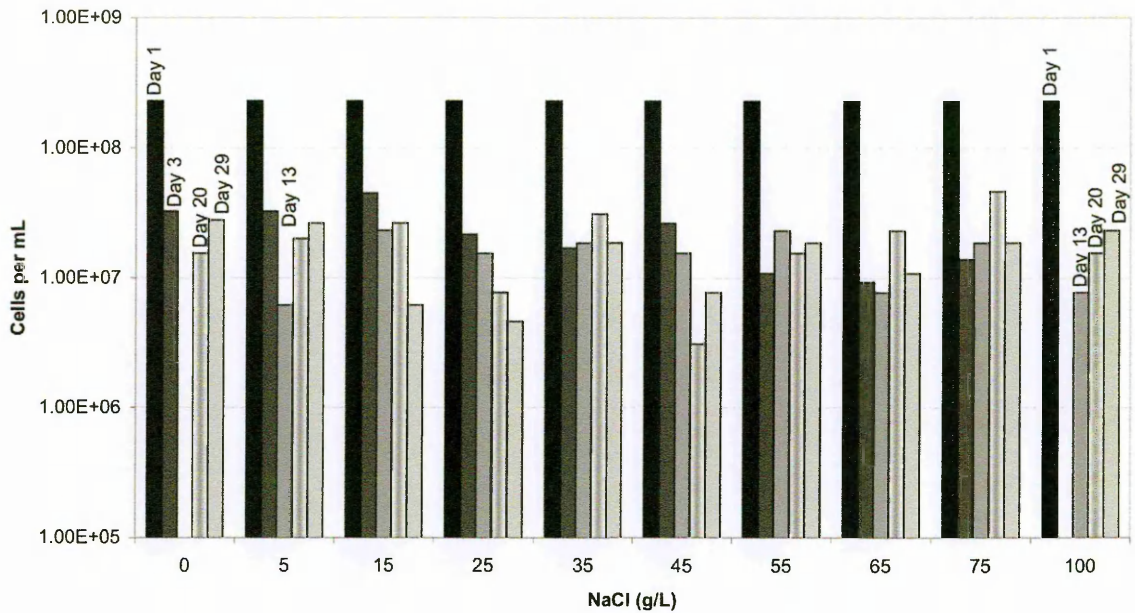
Figure 79 - Optimal NaCl experiment 2 for CBIS 19I1, Experiment 2. Thin bars indicate cultures where cells became clumped into large masses and mats, making it difficult to distinguish individual cells during counts.

Optimal pH

CBIS 19I2 showed little clumping in pH experiments, making cells relatively easy to identify and count. However, varying the pH of solution seemed to have a dramatic effect on the viability of CBIS 19I2. Experiments to optimize pH for CBIS 19I2 were performed in three separate experimental setups and only one produced useable data after an extended period of incubation (212 days, **Figure 80**). In the first two attempts, when bottles were opened to perform cell counts and to test the pH of media, counts were only successful in the first 5-10 days of the experiment (data not shown). After this period, all cells in all replicates at the pH levels tested appeared to die off. For the third attempt, cultures were counted once after 5 days of incubation and then left undisturbed in incubation until the completion of the experiment.

Optimal pH for growth of CBIS 19 I2
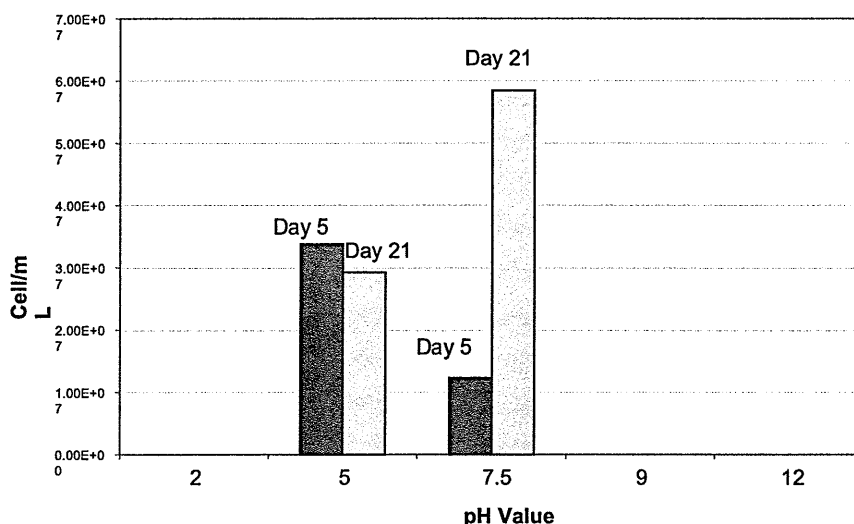
Figure 80 - Optimal pH data for CBIS 19 I2 following 212 days of incubation.

Carbon Source Utilization

The carbon source utilization profile based on results of the Biolog-AN Microplate is

shown in **Table 32** and **Figure 81**. Only four of the 96 wells in the microplate showed a

colour change indicating growth. A weak colour change was seen with D-Fructose and L-

Fructose. A strong colour change was seen with Palatinose and L-Rhamnose.

Water	N-Acetyl-D-Galactosamine	N-Acetyl-D-Glucosamine	N-Acetyl-β-D-Mannosamine	Adonitol	Amygdalin	D-Arabitol	Arbutin	D-Cellobiose	α-Cyclodextrin	β-Cyclodextrin	Dextrin
Dulcitol	i-Erythritol	D-Fructose	L-Fucose	D-Galactose	D-Galacturonic Acid	Gentiobiose	D-Gluconic Acid	D-Glucosaminic Acid	α-D-Glucose	α-D-Glucose-1-Phosphate	D-Glucose-6-Phosphate
Glycerol	D,L-α-Glycerol Phosphate	m-Inositol	α-D-Lactose	Lactulose	Maltose	Maltotriose	D-Mannitol	D-Mannose	D-Melezitose	D-Melibiose	3-Methyl-DGlucose
α-Methyl-D-Galactoside	β-Methyl-D-Galactoside	α-Methyl-D-Glucoside	β-Methyl-D-Glucoside	Palatinose	D-Raffinose	L-Rhamnose	Salicin	D-Sorbitol	Stachyose	Sucrose	D-Trehalose
Turanose	Acetic Acid	Formic Acid	Fumaric Acid	Glyoxylic Acid	α-Hydroxybutyric Acid	β-Hydroxybutyric Acid	Itaconic Acid	α-Ketobutyric Acid	α-Ketovaleric Acid	D,L-Lactic Acid	L-Lactic Acid
D-Lactic Acid Methyl Ester	D-Malic Acid	L-Malic Acid	Propionic Acid	Pyruvic Acid	Pyruvic Acid Methyl Ester	D-Saccharic Acid	Succinamic Acid	Succinic Acid	Succinic Acid Mono-Methyl Ester	m-Tartaric Acid	Urocanic Acid
Alaninamide	L-Alanine	L-Alanyl-LGlutamine	L-Alanyl-L-Histidine	L-Alanyl-L-Threonine	L-Asparagine	L-Glutamic Acid	L-Glutamine	Glycyl-L-Aspartic Acid	Glycyl-L-Glutamine	Glycyl-L-Methionine	Glycyl-LProline
L-Methionine	L-Phenylalanine	L-Serine	L-Threonine	L-Valine	L-Valine plus L-Aspartic Acid	2'-Deoxy Adenosine	Inosine	Thymidine	Uridine	Thymidine-5'-Mono-phosphate	Uridine-5'-Mono-phosphate

Figure 81: Microplate results for CBIS 19I1. Highlighted squares indicate wells where successful colour change occurred. Lighter coloured wells indicate a weaker colour change.

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Carbon Source	CBIS 46
D-Fructose	w
L-Fucose	w
Palatinose	+
L-Rhamnose	+

5.5.6 CBIS 46 I1, Het Med 1

Optimal NaCl

To determine the optimal concentration of NaCl, growth of CBIS 46I1 was monitored in

media containing 0, 5, 15, 20, 25, 35, 45, 50, 60, and 70 g/L NaCl. Three replicates of

cultures at each concentration of NaCl were inoculated with liquid cell cultures yielding an

estimated starting concentration of 1.77×10^6 cells/mL. Cell counts were performed using

the DNA-binding dye DAPI viewed under epifluorescence.

Optimal NaCl Concentration Isolate 46
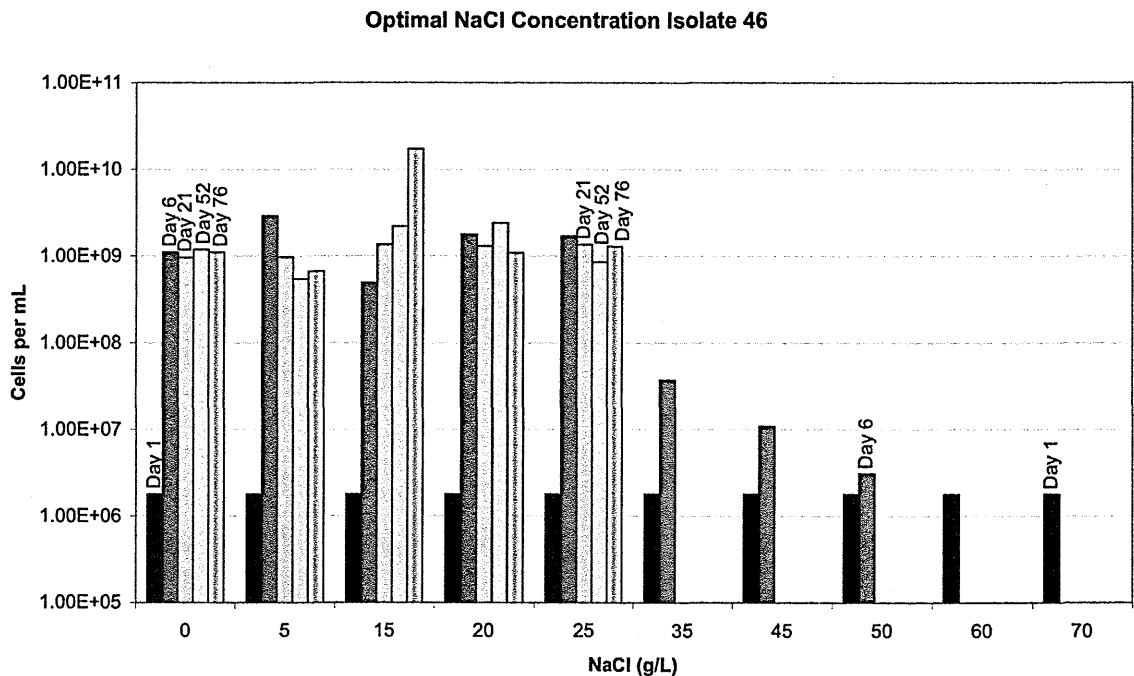
Figure 82 - Growth of CBIS Isolate 46 cultures at varying concentrations of NaCl. Growth was
monitored for 76 days of incubation. Thin bars indicate cultures where cells became clumped into
large masses and mats, making it difficult to distinguish individual cells during counts.

CBIS 46I1 was able to grow in salt concentrations from 0-25 g/L when incubated for 76

days at 37°C. Cultures initially showed growth in concentrations up to 50 g/L NaCl after

incubation for 6 days, but cultures in 35 g/L, 45 g/L and 50 g/L NaCl were not viable after

incubation for 21 days. Final cell counts became difficult due to clumping of cells in large

groups. However, it appeared that growth of CBIS 46 reached the highest levels in solutions containing 15 g/L NaCl.

Cell numbers in these tests increased rapidly and then remained stable after an initial peak. On average, cultures tended to peak in cell numbers at around 8 days and remained at stable levels near 1×10^9 cells/mL through the full incubation period. One culture, at 15 g/L NaCl appeared to continue increasing in cell numbers beyond eight days

Optimal pH
CBIS 46I1was tested in three replicates of solutions of pH 3.0, 6.0, 7.5, 9.0 and 12.0. Cell counts were not performed in this test because the staining efficiency of the DAPI appeared to be extremely low, and counts could not be made with confidence. Instead, cultures had to be marked as a simple 'positive' and 'negative' based on the presence of cells viewed under white light. After 5 days of incubation at 37°C, cells were present in all cultures except for those at pH 12.0. After incubation for 44 days, cultures grown at pH 3.0 had died off, but significant numbers of cells remained present at pH 6.0, 7.5 and 9.0. After 55 days, cultures remained at these pH levels.

Carbon Source Utilization
The carbon source utilization profile for CBIS 46 I1 based on results of the Biolog-AN Microplate are shown in **Table 33** and **Figure 83**. Eight wells out of 96 showed a change in colour after one week of incubation. The strongest colour change was in wells containing D-Fructose, Palatinose and L-Rhamnose. A week colour change was seen in wells containing L-Fucose, D-Galactose, D-Galacturonic Acid, D-Mannose and D-Glucose-6-Phosphate. An additional, very weak colour change was seen in two additional wells (Turanose and Pyruvic Acid) after 36 days of incubation.

Water	N-Acetyl-D-Galactosamine	N-Acetyl-D-Glucosamine	N-Acetyl-β-D-Mannosamine	Adonitol	Amygdalin	D-Arabitol	Arbutin	D-Cellobiose	α-Cyclodextrin	β-Cyclodextrin	Dextrin
Dulcitol	i-Erythritol	D-Fructose	L-Fucose	D-Galactose	D-Galacturonic Acid	Gentiobiose	D-Gluconic Acid	D-Glucosaminic Acid	α-D-Glucose	α-D-Glucose-1-Phosphate	D-Glucose-6-Phosphate
Glycerol	D,L-α-Glycerol Phosphate	m-Inositol	α-D-Lactose	Lactulose	Maltose	Maltotriose	D-Mannitol	D-Mannose	D-Melezitose	D-Melibiose	3-Methyl-D-Glucose
α-Methyl-D-Galactoside	β-Methyl-D-Galactoside	α-Methyl-D-Glucoside	β-Methyl-D-Glucoside	Palatinose	D-Raffinose	L-Rhamnose	Salicin	D-Sorbitol	Stachyose	Sucrose	D-Trehalose
Turanose	Acetic Acid	Formic Acid	Fumaric Acid	Glyoxylic Acid	α-Hydroxybutyric Acid	β-Hydroxybutyric Acid	Itaconic Acid	α-Ketobutyric Acid	α-Ketovaleric Acid	D,L-Lactic Acid	L-Lactic Acid
D-Lactic Acid Methyl Ester	D-Malic Acid	L-Malic Acid	Propionic Acid	Pyruvic Acid	Pyruvic Acid Methyl Ester	D-Saccharic Acid	Succinamic Acid	Succinic Acid	Succinic Acid Mono-Methyl Ester	m-Tartaric Acid	Urocanic Acid
Alaninamide	L-Alanine	L-Alanyl-L-Glutamine	L-Alanyl-L-Histidine	L-Alanyl-L-Threonine	L-Asparagine	L-Glutamic Acid	L-Glutamine	Glycyl-L-Aspartic Acid	Glycyl-L-Glutamine	Glycyl-L-Methionine	Glycyl-L-Proline
L-Methionine	L-Phenylalanine	L-Serine	L-Threonine	L-Valine	L-Valine plus L-Aspartic Acid	2'-Deoxy Adenosine	Inosine	Thymidine	Uridine	Thymidine-5'-Monophosphate	Uridine-5'-Monophosphate

Figure 83: Biolog results for CBIS 46. Lighter coloured wells indicate a weaker colour change.

Table 33 - Carbon source utilization by CBIS 46. Carbon sources marked "w" indicate only a weak colour change in the Biolog plate well. Carbon sources marked "ww" indicate a weak colour change that occurred only after 36 days of incubation at 37°C.

Carbon Source	CBIS 46
D-Fructose	+
L-Fucose	W
D-Galactose	W
D-Galacturonic Acid	W
D-Mannose	W
D-Glucose-6-Phosphate	W
Palatinose	+
L-Rhamnose	+
Turanose	WW
Pyruvic Acid	WW

5.5.7 TEM Imaging of cell cultures

Cell cultures in liquid media and on solid agar plates were examined with Transmission Electron Microscopy (TEM). The cultures included CBIS 16PI, CBIS 16I1, CBIS 19PI, CBIS 19I1, CBIS 46PI and CBIS 46I1. Additionally, TEM imagery was used to examine the sulphide deposits found in Het Med 1 in order to prevent later confusion between these deposits and cultured cells. Sulphide deposits were dark, spherical objects that clustered together in groups.

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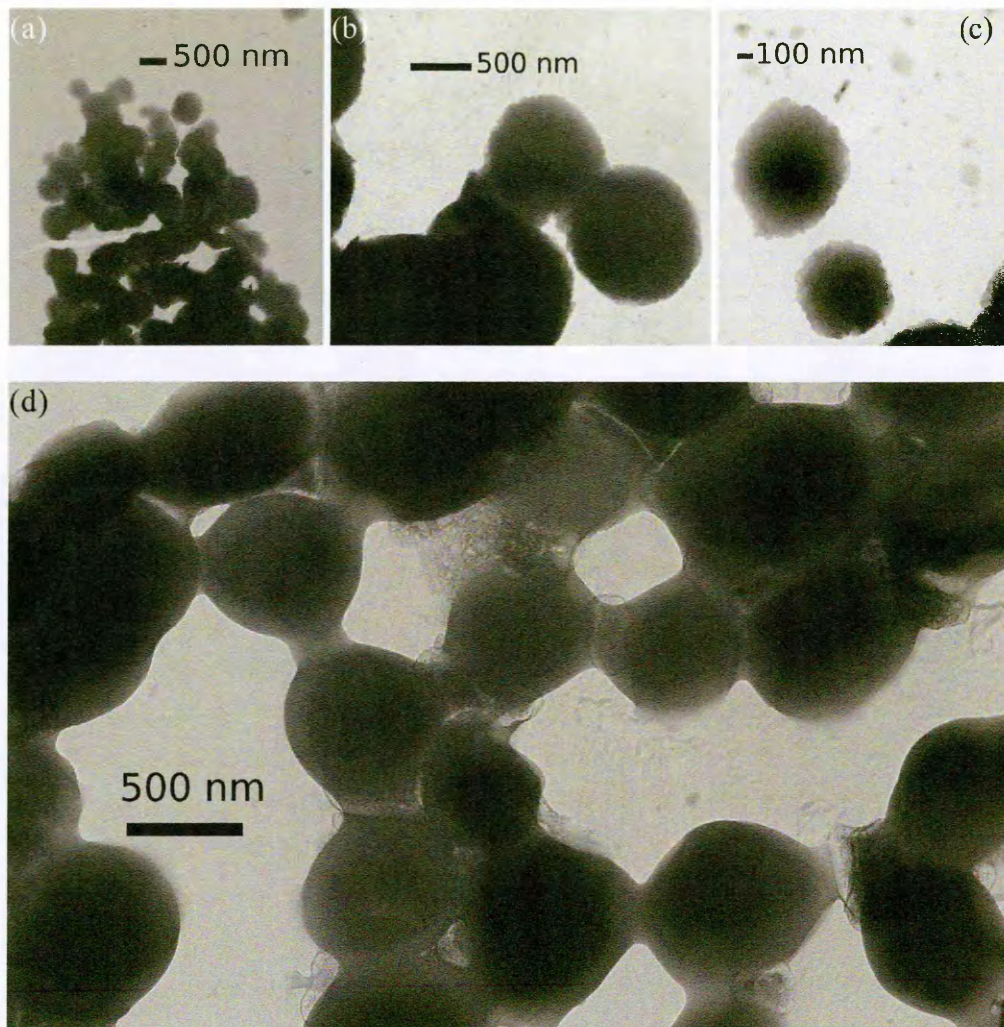
Figure 84 - TEM images at varying magnification of sulphide precipitates in Het Med 1. These precipitates were collected from cultures CBIS 16I2 (a), CBIS 19I1 (b), CBIS 46I1 (c) and a control of sterile Het Med 1 (d).

TEM images collected from CBIS 16I1 were somewhat difficult to interpret. Samples appeared to contain a large amount of non-cellular material in addition to cell-like structures. Distinct rod-shaped cells were seen, as well as cells that could have either been coccoid in shape, or simply rod-shaped cells viewed side-on from the forward or reverse position. Other, large ovular cell-like structures were also present (**Figure 94**).
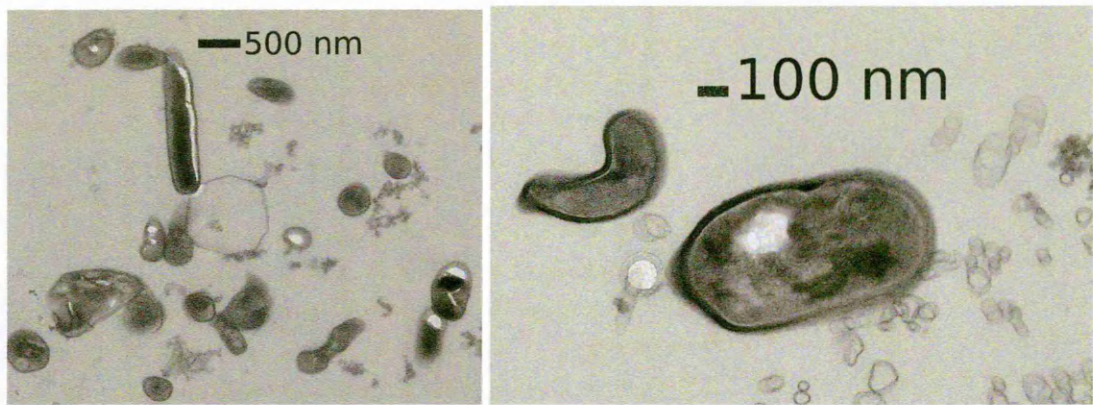
Figure 85 - TEM images collected from cultures of CBIS 16I1.

Similar to CBIS 16I1, plate cultures of CBIS 16PI viewed with TEM showed a large amount of non-cellular material. Plate cultures were dense and cultures were thick and viscous when removed from the plate. Many rod-shaped cells could be seen embedded in mat-like material (**Figure 86**). **Figure 87** shows a close-up view of the rod shaped cells where cell walls are visible.
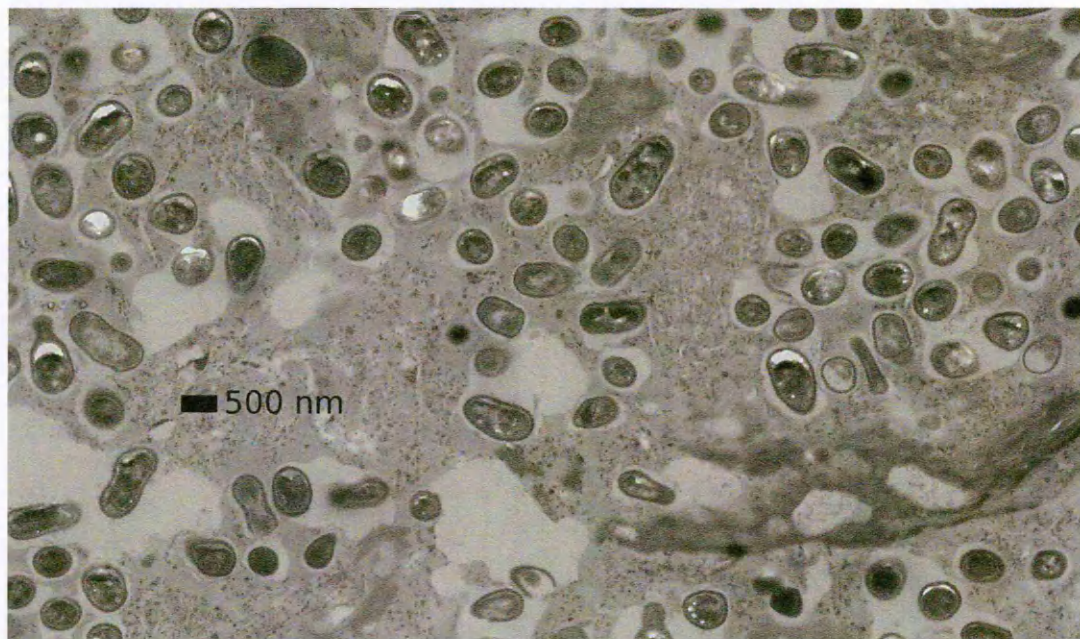
Figure 86 - TEM image from plate culture of CBIS 16PI.
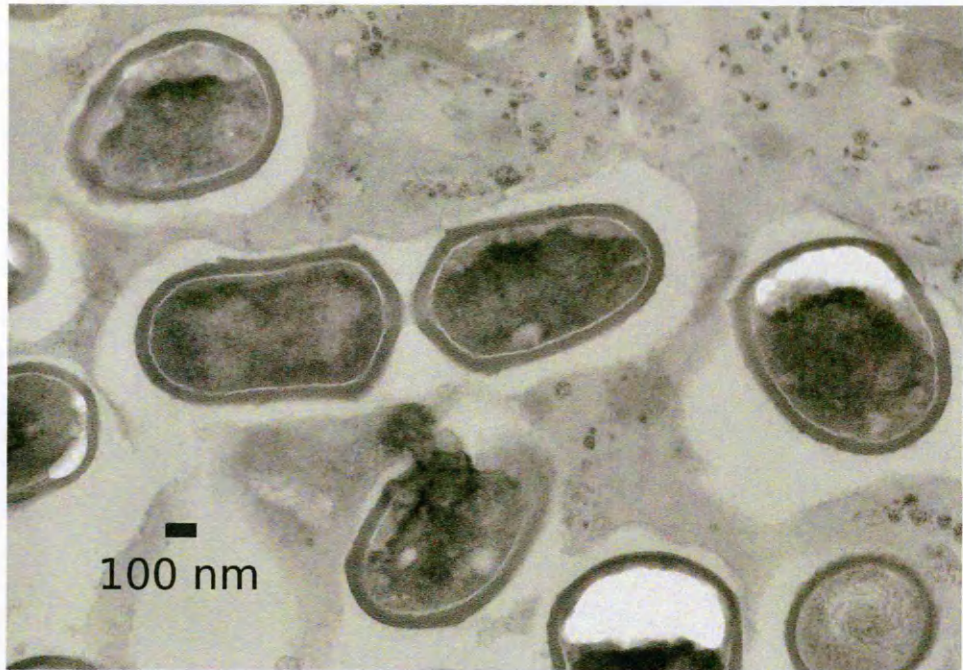
Figure 87 - TEM image from plate culture of CBIS 16PI.
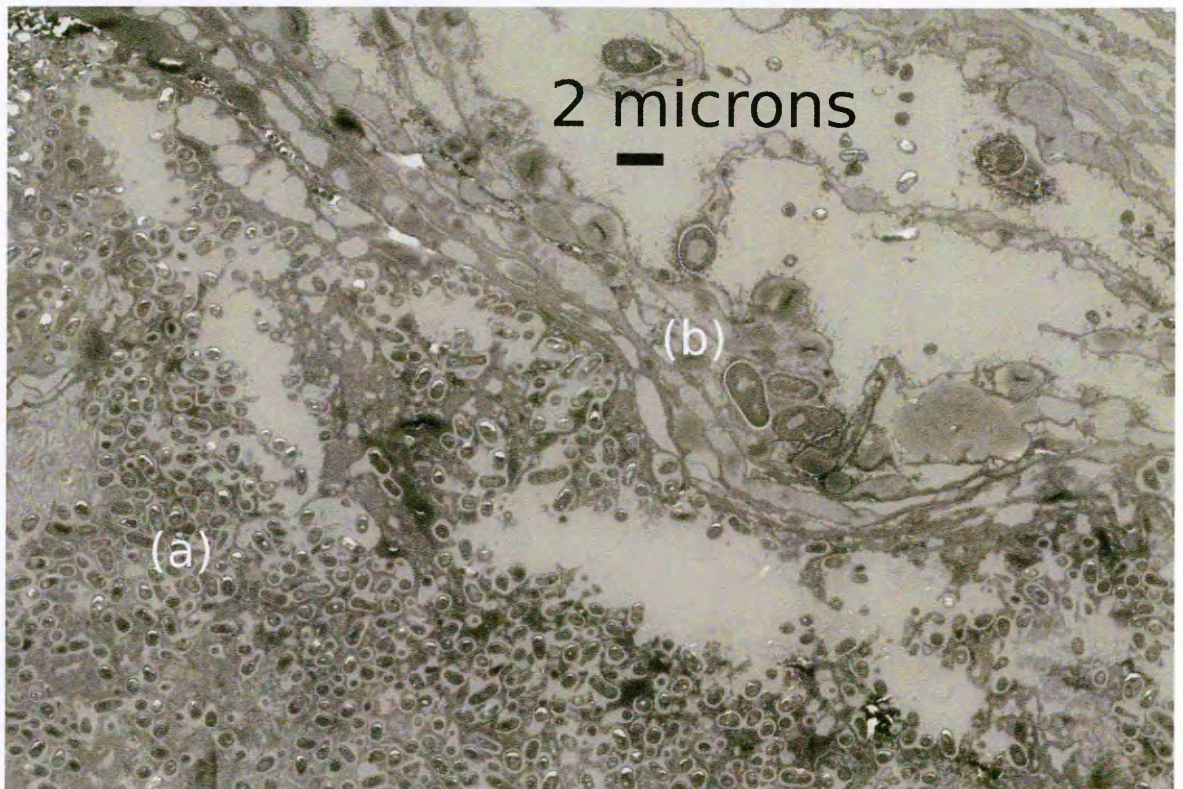
Figure 88 - A wide view of CBIS 16PI where small rod-shaped cells (a) embedded in mat-like material and large, ovular cells (b) outside of mats.

As with CBIS 16I1, cultures also contained large, ovular shaped cell-like structures

(**Figure 88**). The ovular cells had a unique internal structure, which appeared to be almost

a spiral-like series of vesicles (**Figure 89**). No other internal structures could be seen inside

the cells.
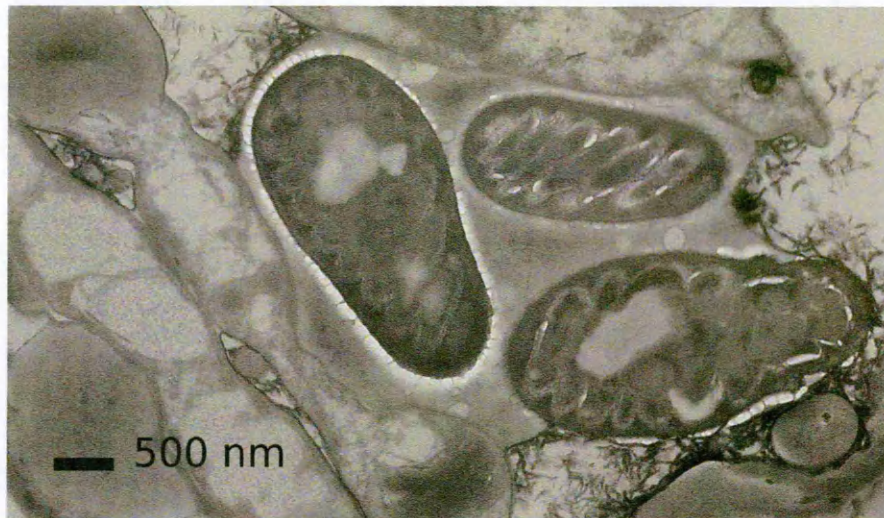
Figure 89 - Close-up image of the large, ovular cells.

As mentioned, the ovular shapes were typically located outside of the mat-like material that encased the rod-shaped cells. However, occasionally a single ovular cell could be found amongst the rod-shaped cells (**Figure 90**).
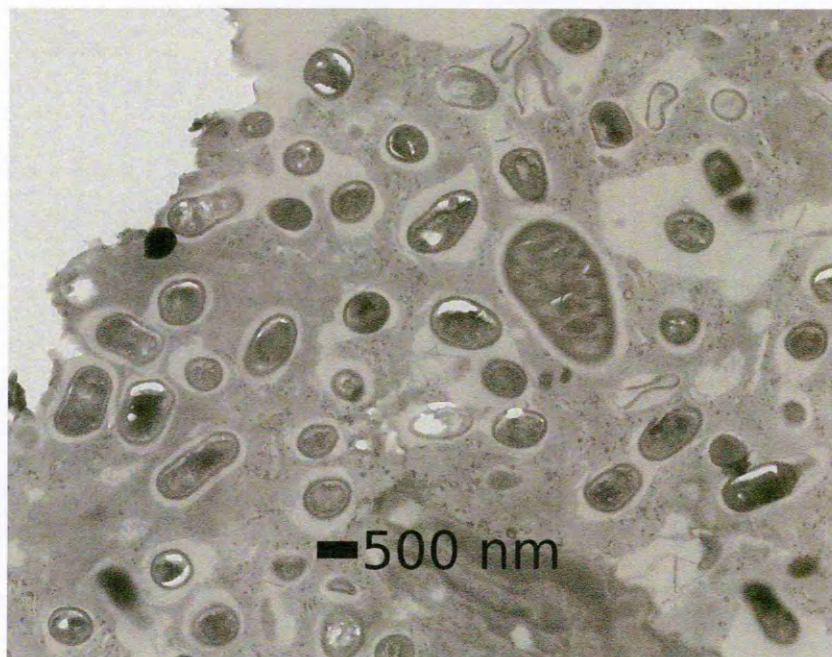
Figure 90 - TEM image of CBIS 16PI.

CBIS 19I1 was composed of rod shaped or coccoid cells depending on their orientation on the slide (**Figure 91**). Some of these cells could be seen dividing or budding off smaller cells (**Figure 92**). Cells had a visible cell wall, and sometimes showed collections of non-cellular material outside of the cell.
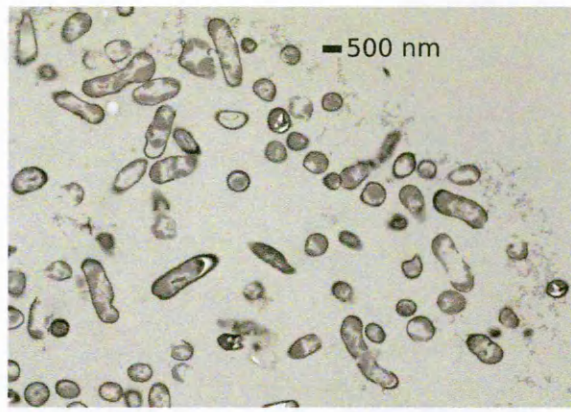
Figure 91 - TEM image of CBIS 19I1.
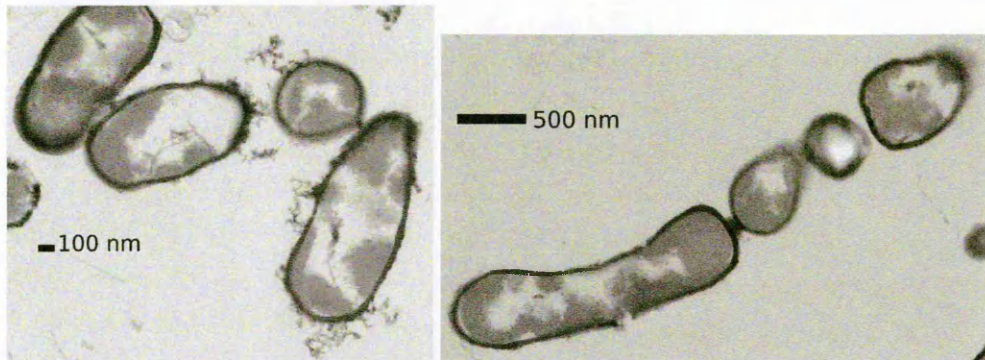
Figure 92 - TEM image of CBIS 19I1.

CBIS 19PI appeared similar to CBIS 19I1, however there was a large amount of non-cellular material, some of it strand-like in nature, outside of the cells (**Figure 93**).
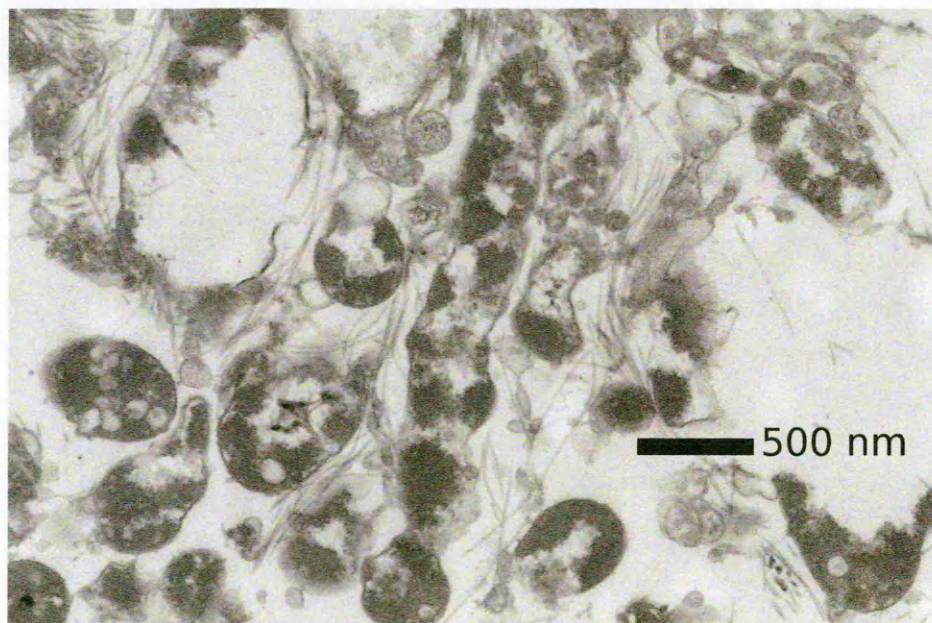
Figure 93 - TEM image of CBIS 19PI.

CBIS 19PI also contained rod shaped cells, which could be seen budding off small cells. In both CBIS 19I1 and CBIS 19PI, many cells were damaged. A large number of 'ghost' cells,

or cells that had ruptured leaving behind only cellular components such as membranes, were present.

As with the previously discussed cultures, CBIS 46I and CBIS 46PI appeared to be composed of identical cell types. Cell densities were higher in CBIS 46PI, as materials in cell cultures on plates are more concentrated. Both cultures also contained the spherical sulphide deposits characteristic of Het Med 1. CBIS 46I and CBIS 46PI contained coccoid cells with a thick cell wall. Dividing cells and ghosts of ruptured cells were present in both cultures.
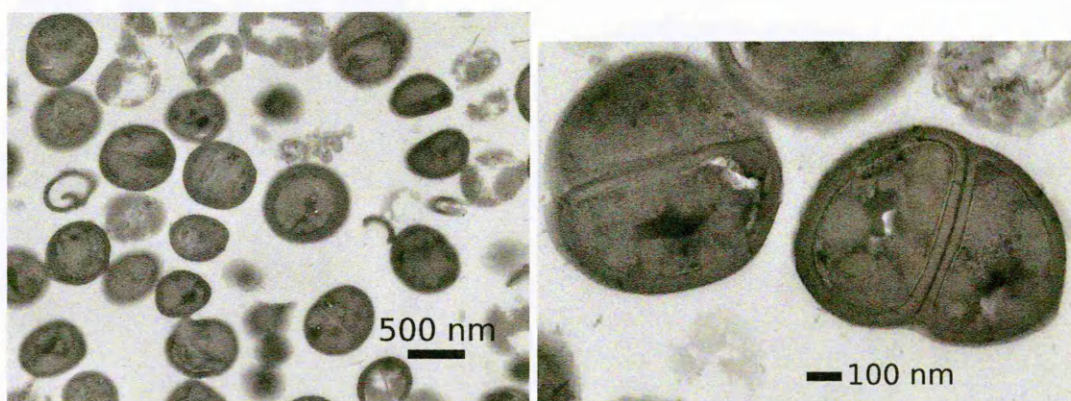
Figure 94: TEM image of CBIS 46 PI.
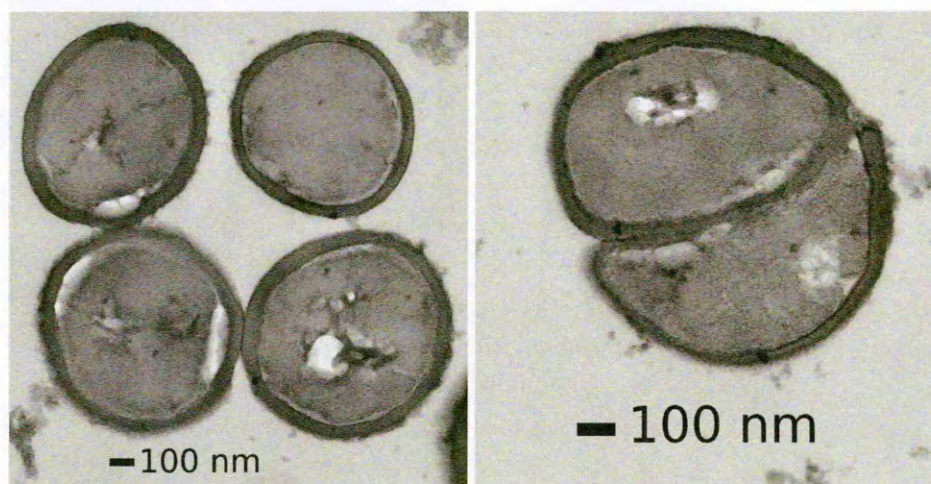
Figure 95: TEM image of CBIS 46I1.

5.6 DISSCUSSION

Previous studies on subsurface environments have returned a range of microorganisms, both specialists and heterotrophs. In studies on sediments from the Savannah River near Aiken, S.C., the deepest sediments (~ 265 meters) sampled actually showed some of the highest densities and diversities of bacteria (Jiménez 1990). These microorganisms

included methanogens, sulfate reducers, nitrate reducers and heterotrophs (Fredrickson and Hicks 1987). Interestingly, the diversity of communities, in particular heterotrophs, did not decrease with depth at the Savannah River site. Additionally, most of the communities proved to be aerobic or facultative chemoheterotrophs with oxidative organisms outnumbering fermentative organisms by 82% to 4% (Jiménez 1990). These communities at much shallower depths than the CBIS contained a number of strains that were unidentifiable by standard biochemical assays.

Obtaining isolate cultures from CBIS enrichments was an important part of identifying unique species present at depth in the CBIS. Isolates were obtained from enrichment cultures from CBIS sediments with both Het Med 1 heterotrophic media and Fe USGS media for iron reducing microbes. In both media types, isolates could be obtained with serial dilution in liquid media, serial dilutions performed in MPN tests and growth of plate colonies on solid media.

As with growth of original enrichment cultures, problems with the growth of isolates were encountered when transferring cells into fresh media. Oftentimes, when setting up a dilution series to obtain isolates, cultures would only grow in the first or second dilution. When isolates were obtained, it was often difficult to keep them alive because, when inoculated into fresh media, cultures would have trouble re-growing. Unfortunately, the difficulty in maintaining cultures meant that fewer isolates than expected could be obtained and kept in stable cultures for study.

The difficulty in maintaining isolates and the inability of isolates to grow in high densities presented challenges in obtaining amplifiable DNA and performing the physiological and chemical tests required to accurately identify species types. Tests for G+C content and fatty acid content of organisms require large amounts of cellular material. Additionally, high concentrations of cells were found to be necessary in order to obtain successful DNA extraction for PCR amplification with many of the cultures. The low cell numbers present and the inability to re-grow cultures or cross-inoculate cells into fresh

media caused severe limitations in the number of tests that could be performed and the number of DNA extractions that proved successful.

In total, seventeen isolates were collected from CBIS enrichment cultures. These isolates represent six separate depths within the CBIS that range from 571.1 m to near the very bottom of the core at 1608.8 m. Both plate spreading and serial dilutions in liquid media were used to obtain isolates, however these isolates were obtained after only a very low number of dilutions. This indicated a low general diversity in enrichment cultures; however, it also made continual culturing of isolates difficult. Isolate cultures often died off quickly and inoculating cells into fresh media rarely produced new cultures.

Because cells tended to die off after only a few dilutions and isolates were difficult to re-grow in fresh media, it is thought that culture media is not fully optimized for growing cultures under laboratory conditions. The organisms obtained are ill-suited for growth in the laboratory, and it is possible that sediments from CBIS cores contain specific nutrients or minerals required for growth. Cultures were easiest to grow in enrichment cultures, which is expected as organisms in natural habitats live in a consortium and their growth is often tied to the successful growth of other members in the colony. However, even enrichment cultures were difficult to re-grow in fresh media when removed from sediments. As cells were diluted through multiple inoculations, the concentration of sediment particles would also decrease. If sediments did indeed contain necessary components for growth, dilution of enrichment cultures would eventually remove these components, or reduce them to negligible levels.

Amplifiable DNA was successfully extracted from only five of these cultures, four of which yielded useable sequences (**Table 34**). Additional sequences were also obtained from DNA extractions from enrichment cultures (**Table 34**). Many of these enrichment cultures appeared to have only single cell-types present, and clean sequences obtained from these cultures indicated that they are dominated by, or potentially composed of, single species types.

Table 34 - Isolate and Enrichment cultures from which DNA extraction was attempted. The size of successful sequences is given in base pairs and the closest matching species type according to the NCBI Blast database is listed. Closest matching species types in clone libraries is also included.

Culture for Extraction	Extraction Type	Amplifi-able Product	Length of Sequence in base pairs	Plate or Liquid Culture	Closest Matching Species Type	Max Identity (%)
CBIS 8 Fe-Citrate	FT Extraction	No	N/A	Liquid	N/A	N/A
CBIS 10E	Ft Extraction	No	N/A	Liquid	N/A	N/A
CBIS 16 I1	FT Extraction	No	N/A	Liquid	*Pseudomonas* sp.	81
	FastDNA Kit	Yes	431			
CBIS 16I2	FT Extraction	No	N/A	Liquid	N/A	N/A
	FastDNA Kit	No	N/A			
CBIS 16I3	FT Extraction	No	N/A	Liquid	N/A	N/A
CBIS 16PI	FT Extraction	No	N/A	Plate	*Jonesia* sp	99
	QIAgen puregene tissue kit	Yes	1342			
CBIS 16E	FT Extraction	No	N/A	Liquid	*Jonesia* sp.	95
	QIAgen puregene tissue kit	Yes	1462			
CBIS 19I1	FT Extraction	No	N/A	Liquid	N/A	N/A
CBIS 19I2	FT Extraction	No	N/A	Liquid	N/A	N/A
CBIS 19I3	FT Extraction	No	N/A	Liquid	N/A	N/A
CBIS 19I4	FT Extraction	No	N/A	Liquid	N/A	N/A
CBIS 19PI	FT Extraction	No	N/A	Liquid	*Bacillus* sp.	98
	FastDNA Kit	Yes	414			
CBIS 19E	QIAgen puregene tissue kit	Yes	1428	Liquid	*Bacillus* sp.	99
CBIS 28ST	FT Extraction	No	N/A	Liquid	*Pseudomonas* sp.	100
	FastDNA kit	Yes	519			
CBIS 28E	FT Extraction	No	N/A	Liquid	Uncultured clones	99
	FastDNA kit	Yes	1007			
CBIS 39-1 E	FT Extraction	No	N/A	Liquid	Uncultured clones	99
	FastDNA kit	Yes	860			
CBIS 39-1 I	FT Extraction	No	N/A	Liquid	N/A	N/A
CBIS 39-1 PI	FT Extraction	No	N/A	Plate	N/A	N/A
CBIS 45E	QIAgen puregene tissue kit	Yes	1511	Liquid	*Trichococcus collinsii*	99
	FT Extraction	No	N/A			
	FastDNA kit	Yes	923	Liquid	Uncultured Clones	98
CBIS 45I	FT Extraction	No	N/A	Liquid	N/A	N/A
CBIS 46PI	FT Extraction	No	N/A	Plate	N/A	N/A
	FastDNA kit	No	N/A			
CBIS 46I	FT Extraction	No	N/A	Liquid	N/A	N/A
CBIS 46E Clone Library	Closest matching species from clone library constructed from CBIS 46E			Liquid	- *Cellulomona* sp. - Uncultured *actinobacter*	N/A

DNA extraction proved most successful with the original enrichment cultures from all depths in the CBIS where cultures were successfully obtained. The largest fragments of DNA, which also provided the cleanest sequences when analyzed using the ABI310 genetic analyzer, were actually obtained from the original enrichment cultures of CBIS 16E, CBIS 19E and CBIS 45E. Typically, enrichment cultures contain an assortment of

cell and species types; and DNA extractions therefore contain a multitude of sequences. A single sequence cannot typically be read using a genetic analyzer in this case.

This was not the case with enrichment samples from the CBIS. DNA extracted from enrichment cultures returned large, amplifiable fragments of DNA that produced very clean sequences in the genetic analyzer. This indicates that only a single species was present in the original enrichment cultures, or that one species was so dominant that the vast majority of amplicons produced in PCR were obtained from this dominant organisms' DNA. This would indicate that all isolates obtained from enrichments were simply dilutions of these single, dominant organisms.

In the case of CBIS 16E, this idea is supported by the fact that a clean sequence was also obtained from the isolate CBIS 16PI. Both sequences from CBIS 16PI and the original enrichment culture CBIS 16E corresponded to a *Jonesia sp*. Similarly, both the enrichment culture CBIS 19E and the isolate CBIS 19 PI returned sequences that were identified as *Bacillus* species, with the closest matching organisms being unidentified *Bacillus* species from marine environments. The sequence returned by CBIS 19 PI was a relatively small fragment (~400 bp) returned from FastKit extractions, and contained a number of base pairs that could not be accurately identified, meaning that the resulting sequence likely contained more errors than the clean, and relatively lengthy (>1400 bp) sequence obtained from CBIS 19E. It cannot be guaranteed, however, that isolate cultures obtained from CBIS 16E and CBIS 19E are associated with the sequences obtained directly from the enrichment cultures, however likely it may appear. It is possible that the isolates represent organisms that are present at very low numbers in the enrichment cultures. Additional attempts to successfully extract amplifiable and sequenceable DNA from isolate cultures must be performed in order to identify the species types of these cultures accurately.

Amplifiable DNA could not be obtained from any of the CBIS 45 isolates by any of the methods attempted. A sequence was successfully obtained from DNA extracted

directly from the original enrichment culture, CBIS 45E. Direct sequencing of this enrichment produced a very clean sequence. This, in addition to the fact that enrichment cultures appeared to contain only one cell type, indicated that the original enrichment cultures of CBIS 45E contained only one species (or one highly dominant species that represented a large majority of genetic material present in PCR product). If multiple species were present, their respective DNA fragments would not have produced a single, readable and clean sequence when analyzed.

However, in MPN studies of CBIS 45E, and additional cell type was briefly obtained (CBIS 45 I4). This indicates that multiple species could indeed be present in CBIS 45E. If the isolates CBIS 45 I1, I2 and I3 represent the same species of organism, it is possible that this organism is dominant in the enrichment cultures obtained with Het Med 1, and quickly out-competes CBIS 45 I4 for nutrients when CBIS 45 I4 is not immediately isolated from the enrichment (as with the MPN tests, where dilutions are performed immediately after inoculating the media with sediments). If this is the case, CBIS 45 I4 and any other less competitive species may have died out of enrichment cultures before DNA was extracted for amplification, leaving a high enough concentration of DNA from the more dominant species (i.e. potentially the organism present in CBIS 45 I1, I2 and I3) to produce amplified DNA fragments that produced a clean sequence.

The enrichment culture, CBIS 46E, was the only enrichment culture that appeared to contain sequences from multiple organisms. Clone libraries were constructed from DNA extracted from CBIS 46E and showed only two groups of organisms corresponding to *Cellulomona sp.* and uncultured *actinobacter* species. This indicates that while multiple species types were indeed present in CBIS 46E, diversity was still quite low.

The species diversity of CBIS cultures was low, evidenced by the ability to obtain clean sequences from enrichment cultures. Additionally, cells from multiple isolates obtained from an enrichment culture typically showed only one type of morphology. Moreover, cells in isolates also resembled cells predominant in enrichment cultures. The

clone library constructed from CBIS 46E at the depth of 1608.8 m indicated only two main groups of organisms (see **Chapter 4**). Species members associated with *Pseudomonas, Jonesia, Actinobacteria, Cellulmona* and *Bacillus* were identified in cultures.

Extraction of DNA from cultures proved difficult, and even when DNA was successfully obtained it could not always be amplified and sequenced. Multiple primers were used for PCR amplification, and for many of the cultures only primers that yielded the smallest fragments were successful in returning readable sequences. Problems have been cited previously with the use of 16S primers such as 27f, 1492r and 1392r when used with DNA extracted from sources such as deep-sea sediments or biofilms associated with stones in lotic habitats (Marchesi *et al.* 1998). DNA from these environments was found to be poor template for amplification (Marchesi *et al.* 1998). Results indicate that this may be true for DNA in cultures from the CBIS as well. Extractions from both liquid and plate isolates showed inconsistent results. For instance, DNA was successfully extracted from CBIS 45E using two separate extraction kits. Ultimately, the sequences obtained yielded varying results in terms of the closest matching sequences (**Table 34**). Isolate extractions yielded amplifiable DNA with a range of primers, however many of the sequences were not readable when analyzed (**Table 28** above).

5.6.1 Isolate Studies

Three isolates were selected for further optimal growth test based primarily on their relative ease to culture. Although these three isolates were the easiest to culture, it was still difficult to grow them across multiple inoculations. Growth tests for optimal temperature failed in all attempts. Optimal pH and NaCl concentration, as well as carbon source utilization were determined for these three isolates.

5.6.2 CBIS 16I2

The first isolate, CBIS 16I2, unfortunately yielded no useable DNA. Sequences obtained from both plate isolates and directly from liquid enrichment cultures indicated that *Jonesia* species were prevalent in cultures from this depth (571.1 m). The closest matching

sequence for CBIS 16E, *Jonesia qinghaiensis*, was isolated from soda lake mud in western China (Schumann *et al.* 2004). This bacteria falls into the order *Actinomycetales*, and cells were shown to rod-shaped and arranged in 'threads' (**Figure 96**). This morphology is essentially similar to CBIS 16I2, where individual rod-shaped cells would collect into large clumps as the density of cultures increased. However, cells of CBIS 16I2 were significantly thinner and more strand-like. It is possible that these strands are composed of individual cells forming 'threads' similar to those seen by Schumann *et al.* (2004), as fluorescence was seen in distinct regions along the strand when cells were stained with DAPI (**Figure 97**).
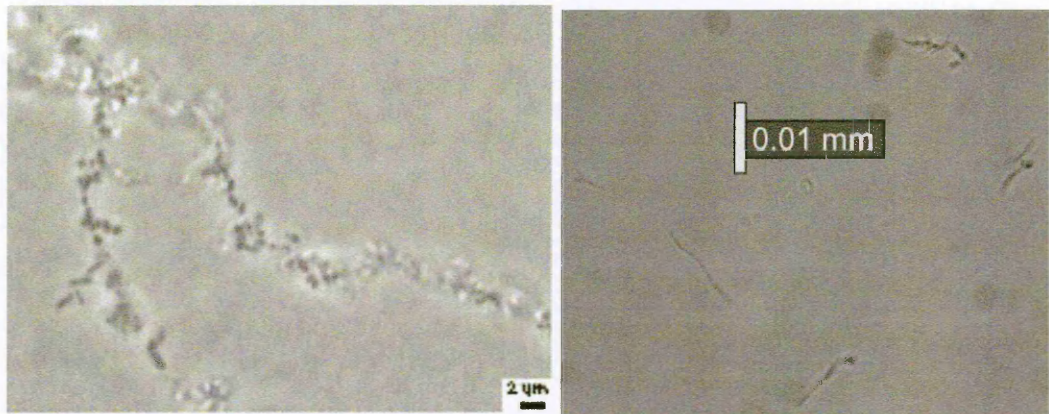
Figure 96 - Micrograph of *Jonesia qinghaiensis* (Schumann *et al.* 2004) as compared to cells of CBIS 16I2 viewed at 1000x magnification under a light microscope.
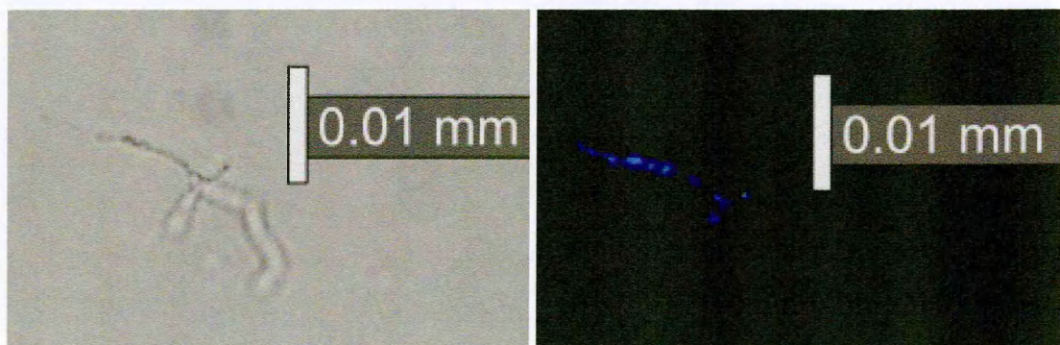
Figure 97 - CBIS 16I2 at 1000x magnification under white light (left) and stained with DAPI (right). When stained, stranded showed multiple pockets of magnification indicating concentrated areas with DNA that could represent individual cells.

The results of carbon source utilization tests for CBIS 16I2 as compared to *Jonesia qinghaiensis* are shown in **Table 35**. The two strains differ significantly in their carbon utilization profiles. *J. quinghaiensis* was able to utilize a far wider range of substrates. D-Fructose was the only carbon source that showed a positive result for both strains.

Additional differences between the two strains were seen in estimated G+C content and

optimal growth conditions

Table 35 - Carbon utilization by CBIS I2 and *J. quinghaiensis* (Schumann *et al.* 2004). Only components tested with both strains are shown.

	CBIS 16	*J. quinghaiensis**
D-Fructose	+	+
L-Fucose	+	-
Palatinose	+	-
L-Rhamnose	+	-
Amygdalin	-	+
D-Cellobiose	-	+
D-Galactose	-	+
D-Gluconic Acid	-	+
D-Melibiose	-	+
glycerol	-	+
D-glucose	-	+
D-mannose	-	+
arbutin	-	+
salicin	-	+
cellobiose	-	+
maltose	-	+
lactose	-	+
sucrose	-	+
trehalose	-	+
dextrin	-	+
maltotriose	-	+
2'-deoxyadenosine	-	+
Methyl pyruvate	-	+

G+C content is based on the estimate drawn from the sequence returned from CBIS

16E, as clean sequence could not be obtained from CBIS 16I2. Because the sequence

drawn from CBIS 16E was a direct DNA extraction from the enrichment, and showed a

high similarity to the small sequence drawn from CBIS 16PI, it is likely that enrichment

cultures at this depth are primarily, or possibly completely, composed of the same

organism cultured in CBIS 16PI. Both sequences showed a high relationship to *Jonesia* sp.

The only other sequence successfully obtained from this depth was from CBIS 16I1, which

showed a low similarity (81%) to a *Pseudomonas* sp. However, the sequence for CBIS

16I1 was small and contained a great deal of uninterpretable base pairs, meaning that the

sequence obtained cannot be considered reliable.

Cell-types were similar for all strains (CBIS 16E, CBIS 16I1 and CBIS 16PI),

being rod-shaped cells that grew in threads and, particularly in the case of CBIS 16I2,

clumps of cells after extended periods of incubation. Both CBIS 16E and CBIS 16PI

strains are also G+C rich based on estimated G+C content (G+C was not determined for

CBIS 16I1 due to low confidence in accuracy of the obtained sequence). The G+C content

range for the genus *Jonesia* (Rocourt *et al.* 1987) is from 56-58%. If estimates based on

CBIS 16E and CBIS 16PI are considered representative of cultures obtained at this depth,

CBIS 16I2 would rest at the bottom of this content range. CBIS 16I2 was also shown to

have a similar optimal pH to *J. quinghaiensis*, with both showing the best growth at

conditions approaching neutral.

Table 36 - Additional properties of CBIS 16I2 as compared to
J. quinghaiensis. Estimated G+C is based on sequences derived
from CBIS 16E* and CBIS PI+.

	CBIS 16I2	*J. quinghaiensis**
Estimate G+C	55.5 %*; 56.3%+	57.3%
Cell-type	Rod-shaped, non-motile	Rod-shaped, non-motile
Optimal pH	6~9	7~9
Optimal NaCl (g/L)	0-35	2.0-7.5
Isolated From	CBIS	soda lake mud

It was not stated by Schumann *et al.* (2004) whether *J. quinghaiensis* was aerobic

or facultatively anaerobic. The closest matching strain, *J. denitrificans* (Rocourt *et al.*

1987), which was used to define the genus *Jonesia*, was defined as a facultative anaerobe.

The use of Biolog-GN Microplates for defining carbon sources for *J. quinghaiensis*

indicates that the organism is aerobic as these plates are designed for use in aerobic

conditions. CBIS 16I2 was isolated, and all growth tests were performed, in anaerobic

conditions. Additionally, CBIS 16I2 showed no growth in aerobic conditions using both

liquid and solid media. The inability to grow CBIS 16I2 in oxic conditions indicates that

the strain is an obligate anaerobe.

Another important difference between CBIS 16I2 and *J. quinghaiensis* can be seen

in optimal NaCl concentration. CBIS 16I2 is far more salt tolerant, surviving at

concentrations up to 35 g/L. The growth range for CBIS 16I2 is probably slightly higher

than this, as the native salt concentration at depths for CBIS 16I2 is 36.9 g/L. CBIS 16I2

also survived for >13 days at concentrations of 45 g/L and cultures failed only after incubations of > 20 days. Further studies at concentrations ranging between 35-45 g/L need to be performed in order to place an exact upper limit on NaCl concentrations for CBIS 16I2. Even so, the optimal concentration for growth of *J. quinghaiensis* fell between 2.0-7.5 g/L. Interestingly, CBIS 16I2 could survive at concentrations lower and greater than *J. quinghaiensis*.

It would appear that CBIS 16I2 might fall into the genus *Jonesia*. However, further study and repeated attempts at acquiring clean DNA sequences from these isolates is required before a definitive genus for CBIS 16 cultures can be determined. With sequence similarities for CBIS 16E and CBIS 16PI of 95% (*J. denitrificans*) and 99% (*J. quinghaiensis*) respectively, species related to *Jonesia* are likely present in sediments at the sample depth for CBIS 16 (571.1 m). A small sequence obtained from CBIS 16I1 showed its closest match with a known organism to be a species of *Pseudomonas*. However, this sequence was only 431 base pairs in length and the similarity between CBIS 16I1 and *Pseudomonas* was only 81%. CBIS 16I1 sequence was derived from a set of FastDNA kit extractions that showed numerous problems during sequencing.

Cells from CBIS 16 isolates show phenotypic similarities to the *Jonesia* strains, and some growth conditions and other characteristics seem similar. However, important differences are present, namely the far wider range of optimal NaCl concentrations for CBIS 16I2 and the inability of all CBIS 16 isolates to grow in aerobic conditions.

5.6.3 CBIS 19I1

As with CBIS 16I2, no useable DNA was obtained from CBIS 19I1. The low diversity of CBIS 19 cultures, and similarities between cell types present in all CBIS 19 cultures indicates that CBIS 19I1 is likely similar to - and potentially identical to – cultures from which DNA was successfully obtained. The largest sequence (1428 bp) was obtained through direct DNA extraction from enrichment culture CBIS 19E and its closest matching sequence at 99% similarity was *Bacillus* sp. PeC11. A second, smaller (414 bp) sequence

was obtained from CBIS 19PI showed its closest match to be *Bacillus* sp. K38T at 98%.

The closest matching sequence with a published reference was *Bacillus* sp. HZBN43

(99%) (Yoon *et al.* 2001). This reference is an isolate obtained from a traditional Korean

food made from seawater blended with various seafoods, which is then left to ferment. The

study showed that microflora present in this fermented food source originated primarily

from seawater and marine organisms (Yoon *et al.* 2001).

The results of carbon utilization tests for CBIS 19I1 as compared to *Bacillus* sp.

HZBN43 are shown in **Table 37**. Neither strain utilized a wide range of carbon sources,

but D-Fructose was the only carbon source shared by both strains.

Table 37 - Carbon utilization by CBIS 19I1 and *Bacillus* sp. HZBN43 (Yoon *et al* 2001). Only components where at least one strain showed a positive result are shown.

	CBIS 19I1	*Bacillus sp.* HZBN43
D-Fructose	+	+
L-Fucose	+	-
Palatinose	+	N/A
L-Rhamnose	+	-
D-glucose	-	+
maltose	-	+
trehalose	-	+
aesculin	N/A	+
glycogen	N/A	+
starch	N/A	+

Additional differences were apparent in optimal growth tests and estimated G+C content.

CBIS 19 cultures were G+C rich based on estimates derived from 16S rDNA sequences.

Conversely, *Bacillus* sp. HZN43 had a very low G+C estimate of 41%. Both strains were

composed of rod-shaped cells; however there are dramatic differences in their structure

and growth behaviour. *Bacillus* sp. HZBN43 is composed of rod-shaped cells that are

motile by use of a peritrichous flagella and are capable of forming endospores (Yoon *et al.*

2001). CBIS 19I1 is also composed of rod-shaped cells, however there is no indication of

motility or endospore formation. The pH ranges in which the strains are able to survive are

similar, but CBIS 19I1 has a far wider range of growth in concentrations of NaCl. In fact,

CBIS 19I1 showed survival over long incubation periods in concentrations upwards of 100

g/L. Growth of *Bacillus* sp. HZBN43 was inhibited above 14 g/L NaCl. Additionally,

growth of CBIS 19I1 did not occur in either liquid or solid culture in aerobic conditions

whereas *Bacillus* sp. HZBN43 was shown to be a facultative anaerobe.

Table 38 - Additional properties of CBIS 19I1 as compared to *Bacillus* sp HZBN43 (Yoon *et al.* 2001). Estimated G+C is based on sequences derived from CBIS 19E* and CBIS 19PI[+].

	CBIS 19I1	*Bacillus* sp. HZBN43
Estimate G+C	56.2 %*; 55.2%[+]	41%
Cell-type	Rod-shaped	Rod-shaped, motile by flagella, spore forming
Optimal pH	5-7.5 (inhibited <5)	7-8 (inhibited <5)
Optimal NaCl (g/L)	0-100	<14
Isolated From	CBIS	Fermented food product
Oxygen tolerance	anaerobic	Facultative anaerobic

Studies on sequence data from CBIS 19 cultures suggest a phylogenetic affiliation with

Bacillus. Closest matching sequences of known organisms for both CBIS 19 sequences

obtained indicated no other relation than *Bacillus*; and the many of the related sequences

were from studies on marine environments or soils. The only matching sequence from a

described organism showed a number of important differences in physiological profiles

and other characteristics. A defining feature of *Bacillus* species is their ability to form

endospores. Further work is required before CBIS 19 cultures can truly be attributed to

Bacillus.

5.6.4 CBIS 46I

Again, no sequence directly from CBIS 46I could be obtained. DNA that was extracted

from CBIS 46E was used to construct a clone library to provide a cross-section of

community members present in enrichments at CBIS sample depth 46 (1608.8 m)

(**Chapter 4**). Two sets of cultures were identified within the clone library, one

corresponding to *cellulomona* and the second filled with uncultured clones with no

identification other than *actinobacter*. Properties associated with growth of CBIS 46I are

provided in **Table 39**. Optimal pH for CBIS 46I was similar to other CBIS isolates.

Optimal NaCl was lower than other CBIS isolates, which is expected as concentrations of

NaCl native to depths for CBIS 46I were in the range of 23 g/L. Like other CBIS isolates, CBIS 46I was shown to be strictly anaerobic.

Table 39 - Additional properties of CBIS 46I.

	CBIS 46I
Cell-type	Coccoidal, non-motile
Optimal pH	6-9
Optimal NaCl (g/L)	0-25
Isolated From	CBIS
Oxygen tolerance	anaerobic

CBIS 46I had a wider range of utilizable carbon sources when compared to CBIS 16I2 and CBIS 19I. Sources that CBIS 46I was uniquely able to utilize included D-Galactose, D-Galacturonic Acid, D-Mannose, D-Glucose-6-Phosphate, Turanose and Pyruvic Acid.

Table 40 - Carbon source utilization by CBIS 46. Carbon sources marked "w" indicate only a weak colour change in the Biolog plate well. Carbon sources marked "ww" indicate a weak colour change that occurred only after 36 days of incubation at 37°C.

Carbon Source	CBIS 46
D-Fructose	+
L-Fucose	W
D-Galactose	W
D-Galacturonic Acid	W
D-Mannose	W
D-Glucose-6-Phosphate	W
Palatinose	+
L-Rhamnose	+
Turanose	WW
Pyruvic Acid	WW

Without sequence data from CBIS 46I, or related CBIS 46 cultures, it is difficult to classify CBIS 46I with any confidence.

5.7 Summary

Isolate cultures were obtained from a variety of depths throughout the CBIS core; however, these cultures proved difficult to maintain and study in the laboratory. When cultures were successfully isolated from enrichments, they oftentimes died after a short period of incubation and were unable to re-establish in fresh media. The challenges associated with growing isolates caused severe limitations when attempting to determine optimal growth conditions and identification of microbial species present. Molecular

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studies on isolate cultures also proved quite difficult. DNA extractions using a number of commercial kits and techniques often returned DNA that could not be amplified by PCR, or that was un-sequenceable following amplification.

It is likely that the majority of organisms present at depth in the CBIS are uncultureable by standard laboratory techniques. Even enrichment cultures obtained from cores (see **Chapter 4**) appeared to be composed of single cell types. When DNA was extracted from enrichments and directly amplified, clean sequences could be obtained. This was a further indication that enrichments were composed of either single species types, or that a single species was highly dominant in the enrichment and any additional species were present in such low numbers that their DNA did not interfere during sequencing.

In order to identify isolate species with high confidence, additional work is required to determine the optimal growing conditions; and DNA extraction techniques need to be optimized in order to obtain sequenceable DNA.

Chapter 6: Chapter 6: Biological Processing of Meteoritic Material for Microbial Growth

6.1 ABSTRACT

Studies on the microbiology of the CBIS showed that impact events are not wholly devastating for life - even in the case of large-scale impacts that can cause massive geological convulsion in both surface and subsurface environments. At regions near the point of impact, where the release of kinetic energy is greatest, target materials that suffered exposure to powerful compression, fracture and thermal energies associated with the impact are still capable of supporting microbial life after re-colonization. Moreover, the force of impact can serve to create new habitats for life that did not exist prior to the impact event, opening fractures and pore spaces in which new microbial communities can live. Impacts serve to re-distribute materials, including minerals and nutrients important for microbial growth, at depth in the subsurface. Typically, these nutrients and other elements essential for the survival of microbial communities become depleted with depth. Impacts can excavate nutrient-poor materials and then act to 're-shuffle' the materials and re-distribute nutrient stores.

Additional work was conducted at the Open University as part of my PhD research program to examine another aspect of impacts on microbial ecology on Earth. This work was not focused on the affects of impact on Earth-based nutrient sources, but instead examined the ability of microorganisms to utilize the actual material in the impactor itself to provide nutrients and energy. Growth experiments were performed using iron-oxidising bacteria to assess the ability of microorganisms to derive useable iron from both iron meteorites (York and Casas Grandes) and carbonaceous chondrites (Murchison and Cold Bokkveld). Growth experiments were performed in both aerobic and anaerobic conditions. Rapid, biologically-mediated alteration of meteoritic materials was observed in these tests, and it was shown that iron from extraterrestrial material could support the growth of microbial cultures with both meteorite types. This work has implications in understanding

the connections between the biosphere of Earth and inputs of materials from beyond our planet, as well as the potential for life on the early Earth to access minerals from extraterrestrial material.

6.2 INTRODUCTION

Large-scale impacts, such as the one that formed the CBIS, are not a common occurrence on Earth. Early in our planet's history, some ~4 billion years ago, such impacts were likely as much as a thousand times more frequent than they are today (Chapman 2004). In current times, however, these large-scale geological events can be considered rare (Shoemaker 1983). In contrast, small-scale impacts from meteorites and micrometeorites have occurred almost constantly throughout our planet's history and continue to take place regularly today. Estimates of the total amount of extraterrestrial material falling to Earth are in the range of 20,000-40,000 tons per year (Love and Brownlee 1993). Micrometeorites and interplanetary dust particles compose the majority of this material, but impacts of more substantial meteorites are not uncommon.

Studies of the CBIS provided a unique view of how a large-scale impact event alters the subsurface habitat of microbial communities. To examine the affects of impact on microbial life on Earth from another angle, a series of experiments was also performed to assess the ability of microorganisms to utilize meteoritic material as a source of nutrients and energy. Much of this work was part of a larger project examining the affects of microbial activity on weathering of meteorites, and which has been accepted for publication (Gronstal *et al., 2009 (In Press)*). Here I will discuss only the experiments I was directly involved in as part of the larger scope of the project, some of which is additional work that was not published in Gronstal *et al., 2009 (In Press)*.

Microorganisms are able to use metals in various oxidation states as sources of electron donors and acceptors for growth. They are also capable of utilizing compounds like phosphorus as a source of nutrients (Ehrlich 2002). Meteorites carry both metals and other biologically-useful compounds to Earth, and it is expected that microbial

communities would act upon meteoritic material once it is delivered into their habitat. This action from microorganisms could represent an important category in the alteration of materials arriving from extraterrestrial sources, and it is important to understand this process in order to distinguish between biological and purely chemical weathering of such materials. This is particularly important in cases such as the martian meteorite ALH 84001 where controversial claims of martian biological involvement in weathering have been discussed (McKay *et al.* 1996).

Studying the direct effects of microorganisms on meteoritic material also provides another link between the processes of life on Earth and exogenous materials from the space environment. This is interesting in terms of the modern Earth; and is particularly important in terms of the delivery of accessible nutrients and energy to the early Earth at a time when life was first establishing itself. The early Earth suffered a much higher rate of bombardment from extraterrestrial material, with some estimates reaching as much as 200 times greater than the rate of bombardment today (i.e. Love and Brownlee 1993, Hartmann *et al.* 2000). This high rate of impact is thought to have had devastating consequences for life at the planet's surface (Nisbet and Sleep 2001); however, as evidenced by studies of the CBIS, impacts can also provide benefits for microorganisms that inhabit the subsurface of the planet. Despite the destruction potentially reaped at the surface (Sleep *et al.* 1989), meteoritic impacts may have contributed raw materials to the Earth's surface (Jenniskens *et al.* 2000, Chyba and Sagan 1992) that would have been useful for life capable of surviving impacts. This material may have even been essential to the origin, establishment and evolution of microbial life early in the Earth's history.

In our study of microbial interactions with meteoritic material, two types of meteorites were examined: carbonaceous chondrites and iron meteorites. The majority of the growth tests in our study (and those that are discussed in this paper) were performed using iron meteorites, which are composed primarily of alloys of nickel and iron with only minor contributions from carbon and phosphorus-containing minerals (Buchwald 1977).

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Iron meteorites are rarer than carbonaceous chondrites, but they are also more resistant to weathering and can survive longer once they've arrived at the Earth's surface. Carbonaceous chondrites have been shown to contain organic material, such as amino acids, carboxylic acids and sugar-related compounds (Sephton 2002). In addition, these meteorites can contain phosphorus-bearing (Pasek 2007) and nitrogen-bearing (Pizzarello et al. 2006) compounds and inclusions, as well as iron present as anhydrous phases (e.g. olivine) or hydrous phases (e.g. magnetites) (Brearley and Jones 1998). The use of carbonaceous chondrites to support organismal growth has been demonstrated by germinating plant tissue cultures (Mautner 2002). This study showed that major nutrients for growth of plants can be obtained from carbonaceous chondrite material.

Tests on plant tissue growth using materials from carbonaceous chondrites by Maunter (2002) were used to study the potential for utilizing extraterrestrial materials to support human exploration of space, and not specifically focused on how life might interact with extraterrestrial material arriving at the Earth. Colonization of meteorites at the Earth's surface has previously been shown (Steele et al. 2000); and in the case of the Tatahouine meteorite from the Sahara Desert, the microbial community reflected that observed in the surrounding soil (Benzerara et al. 2006). Additionally, a report by Gonzalez-Toril et al. (2005) showed some suggestion that the iron-oxidizing microorganism, Leptosprillum ferrooxidans, could be grown on iron meteorites.

In work at the Open University, the ability of microorganisms to utilize meteoritic material for growth was examined in both aerobic and anaerobic conditions. Here I will focus on the aerobic experiments performed myself, which are more relevant to geologically modern meteorite impacts (See Gronstal et al., 2009 (In Press) for detailed results of anaerobic tests). Microorganisms were grown using only meteoritic material from iron meteorites as a source of half-reactions for growth. Attempts were made to examine the methods used by microbial cells to access elements present in the meteorites in order to produce energy or obtain necessary nutrients for growth. Sterile fragments of

meteorites were used for these tests in order to simulate the ability of specific microorganism types to utilize materials freshly arrived at the Earth's surface prior to contamination with terrestrial organisms and carrying no indigenous microflora.

Experiments were also performed to examine the ability of *T. ferrooxidans* to access additional nutrients from meteoritic material. As mentioned, the Cape York and Casas Grandes meteorites also contain phosphorus at ~1.4 mg/g and ~1.5 mg/g respectively. Phosphorus is an essential chemical element for all living organisms, helping to form cellular components like DNA and RNA and energy-storing molecules like adenosine triphosphate. Phosphorus is often considered limiting in ecosystems, meaning that the amount of phosphate present often dictates the productivity of the biological community. In theory, meteorites and other impactors could provide an input of phosphorus into biological systems if organisms are able to access phosphorus-bearing minerals present in exogenous materials. In this way, a steady input of phosphorus from impacts could provide an essential nutrient for ecosystem growth - an input that could have been particularly important on the Earth before a highly productive biosphere was established to aid in the global cycling of phosphorus on our planet (Cockell, Open University Press - course book; Pasek *et al.* 2007).

6.3 METHODS

Studies were performed in aerobic conditions on iron meteorites (Cape York and Casas Grandes) using the iron oxidizer *Thiobacillus ferrooxidans* (aka, *Acidothiobacillus ferrooxidans*, DSM 583) obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, Germany. *T. ferrooxidans* is a strictly aerobic, gram-positive, rod-shaped and thermophilic acidophile bacteria that grows at optimal temperatures of 45-50°C and at pH of 1.5 to 2.5. Some species are able to grow at lesser temperatures and neutral pH. In the experiments discussed here, growth temperatures were maintained at ~27°C, as recommended by the DSMZ for the strain being used (DSM 583). The growth media for these organisms was prepared according to

media recommended by the DSMZ. This consisted of a modified K medium at 28°C (Silverman and Lundgren 1959) prepared from equal proportions of two stock solutions. Solution A contained 3 g NH_4SO_4, 0.1 g KCl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.01 g $Ca(NO_3)_2$, 1 mL 10N H_2SO_4 in 500 mL ddH_2O. Solution B served as the source of reduced iron and was prepared as 44 g $FeSO_4.7H_2O$ in 500 mL ddH_2O. Solution B was filter sterilized to avoid oxidation of the iron during autoclaving. Solutions A and B were mixed at a 50:50 ratio. In experiments where iron content of the media was replaced with meteoritic material, Solution B was removed from the media and replaced with an equal volume of filter-sterilized dd H_2O.

6.3.1 Meteoritic Materials Used

With the selection of iron-oxidizing cultures as experimental subjects, iron meteorites were used in the majority of the tests to examine the ability of microorganisms to utilize meteoritic material for growth. The two iron meteorites selected (both classified as IIIAB) were Casas Grandes and Cape York. The Smales Collection provided both of these samples, and meteorites were split in a Class 100 clean room and rinsed with dichloromethane:methanol solvent mix to remove terrestrial contamination. Casas Grandes was discovered in 1867 in Mexico, but it has been determined that the meteorite landed on Earth more than 2300 years ago (Sprenkel 1959, Suess and Wänke 1962). Casas Grandes contains an estimated 1.5 mg/g of phosphorus in addition to metallic alloys (Moor *et al.* 1996). The Cape York meteorite was discovered in 1818 in West Greenland, but is estimated to have arrived roughly 1000 years earlier (Nishiizumi *et al.* 1987). Cape York contains 1.4 mg/g phosphorus in additional to metallic alloys (Moore *et al.* 1969).

6.3.2 Schreibersite

The ability of *T. ferrooxidans* to utilize phosphide from schreibersite $((Fe,Ni)_3P)$ - a phosphide bearing mineral found in iron-nickel meteorites - was examined. Because the amount of meteoritic material available for study was limited, meteoritic schreibersite was replaced with an analogue iron phosphide (Fe_3P) metal for these experiments.

Growth media for schreibersite experiments was adapted from the standard *T. ferrooxidans* media outlined above, and in which sources of phosphorus were removed and replaced with Fe_3P. Solution A was prepared by removing the 0.5 g K_2HPO_4 to provide a phosphorus-free control. Solution B was prepared as above, and the two solutions were then mixed 1:1 to produce the final growth media. A third experimental solution, Solution C, was prepared identically to Solution A except that the 0.5 g K_2HPO_4 was replaced with 0.571g Fe_3P. Solution C was mixed and autoclaved prior to addition of Fe_3P.

6.3.3 Enumeration of Cell Numbers

Total cell numbers were estimated in cultures to monitor growth during incubation periods. Aliquots of cultures were removed and stained using the DNA-binding dye Syto 9 according to the manufacturer's instructions (Invitrogen, Paisley, UK), and as outlined for similar cell counts in **Chapters 4** and **5**. Acidic conditions were found to interfere with the staining efficiency of Syto 9 in initial counts, and cells had to be washed with dd H_2O on a 0.2 μm polycarbonate filter prior to staining (See **Chapter 3** for details on polycarbonate filter procedures). Stained cells were viewed under a Leica DMRP microscope equipped with epifluorescence (Leica Microsystems, Bensheim, Germany); and showed green fluorescence using an excitation waveband of 450-490 nm (Leica filter cube I3 and an emission long band cut off filter of >515 nm. Cells from four separate 10μL aliquots were counted for each flask in 50 fields of view to provide an estimate of cell numbers contained in culture.

6.3.4 Experiment Protocol

Samples of the iron meteorites were prepared as above, then broken into small pieces and weighed. All meteorite fragments were flame-sterilized before they were added to experimental flasks. On average, meteorite pieces were in the range of ~0.6 g. Meteorite fragments were placed into 50 mL of diluted solution A in 100 mL Erlenmeyer flasks.

For each experiment, 400 μL of suspended cells from stock cultures was washed by centrifugation at 10,000 g twice in filter-sterilized dd H_2O at 21°C and then added to

experimental flasks or tubes. The concentration of suspended cells in 400 μL was in the range of ~1 x 10^7 cells/mL. All experiments were incubated at 28°C and repeated in three replicates.

Each replicate experiment was set up to include included one experimental flask and four controls. The controls included: 1) Abiotic control: A flask containing Solution A and meteoritic material with no *T. ferrooxidans*, which was used to monitor the abiotic alteration of meteoritic material exposed to the growth medium in the absence of microorganisms. 2) Iron-free control: A flask containing Solution A and *T. ferrooxidans* with no meteoritic material or source of iron (Solution B), which showed that *T. ferrooxidans* requires a source of iron to grow. 3) Standard culture: A flask containing Solution A, Solution B and *T. ferrooxidans* to show that Solution A with a non meteoritic iron source present supported microbial growth. This control also confirmed that the cultures used in the experiment were viable and grew under their standard growth conditions. 4) A flask containing only Solution A and Solution B with no microorganisms present, which provided a comparison of abiotic oxidation of Fe^{2+} with control (3).

6.3.5 Experiments with Schreibersite

Schreibersite experiments were prepared as above using pre-autoclaved 25 mL bottles (**Figure 98**). Controls for the experiments included: 1.) Abiotic control - bottle containing Solution B and Solution C with no organisms, which was used to monitor iron in the growth solution with FeP_3 present (Bottle 2,). 2.) Standard culture: a standard growth solution prepared with a 1:1 mix of the original Solution A (containing phosphorus), Solution B and *T. ferrooxidans*. This was to show the viability of cultures used in the experiment under their standard growth conditions (Bottle 3,). 3.) Abiotic control containing the original Solution A (with phosphorus) and Solution B. This was to monitor iron in the standard growth media in the absence of organisms (Bottle 4). 4.) Phosphorus-free control: a solution containing Solution A (without phosphorus), Solution B and *T. ferrooxidans* (Bottle 5). This was used to test the ability of *T. ferrooxidans* to grow in the

absence of phosphorus. The experimental bottle was prepared by adding Solution C

(containing the schreibersite analogue FeP_3), Solution B and *T. ferrooxidans* (starting

concentration of 5.36 x 10^1 to 5.9 x 10^1 cells/cm^3).
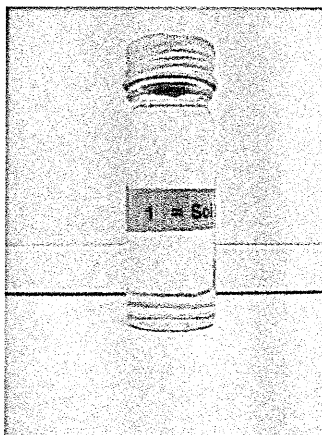
Figure 98 - 25 mL bottle used for Schreibersite experiments.

6.3.6 Ferrozine Assay

The oxidation of extraterrestrial reduced iron by organisms was monitored using a Fe(II)-

specific spectrophotometric assay using ferrozine (4,4'-(3-(2-pyridinyl)-1,2,4-triazine-5,6-

diyl) bisbenzenesulfonic acid, disodium salt) (Stookey 1970). Ferrozine is a compound that

forms a complex with ferrous iron that is magenta in colour and has an absorption peak at

~562 nm. Ferrozine is a standard reagent used as an indicator of ferrous iron, and the

ferrozine method is a standard assay used to measure changing concentrations of iron in

solution. The method is able to identify oxidation of iron, which can be used as a proxy for

growth of organisms in cultures. It should be noted that organisms can oxidize iron without

actually reproducing, so this method provides only a proxy for growth and is not a

conclusive indicator. The ferrozine assay can also be used to monitor abiotic oxidation of

Fe^{2+}, and was used to provide a control Fe^{2+} oxidation rate for abiotic oxidation so that the

actual rate of biologically induced oxidation could be determined. The ferrozine method

also involves a reduction step, which provided a means of determining how much oxidized

Fe was present in solution after the completion of the experiment, as well as the amount of

oxidized iron that was removed from solution as precipitates.

For our experiments, a modified ferrozine method was used to monitor concentrations of Fe(II) in solution over the incubation period for experiments, as described by Viollier *et al* (2000). A stock solution of ferrozine (3-[2-Pyridyl]-5,6-diphenyl-1,2,4-triazine-4,4'-disulfonic acid Na-salt) was prepared in ammonium acetate (10^{-1} mol/L; 0.38 g ammonium acetate in 50 mL H_2O adjusted to pH 9.9) to a concentration of 0.01 M. A reducing agent, 1.4 M hydroxylamine hydrochloride ($H_2NOH.HCl$), was prepared by adding 0.97 g hydroxylamine hydrochloride to 10 mL 2 M HCl. Finally, a buffer solution was prepared by adding 7.7 g ammonium acetate to 10 mL H_2O and adjusted to pH 9.9 using 30% ammonium hydroxide.

The initial concentration of reduced iron (A_1) present in solution was determined by adding 100 µL of ferrozine solution to 900 µL of sample in a cuvette and measuring the absorbance at 562 nm by use of a spectrophotometer (Helios Spectrophotometer, Thermo Scientific, UK). After taking the reading, 800 µL of the solution was transferred to a clean cuvette and 150 µL of the reducing agent was added. Reduction of Fe(III) to Fe(II) was then allowed to occur over a period of 10 minutes. Following this, buffer solution was added and the absorbance (A_2) corresponding to the total iron (Fe(II) from soluble Fe(II) and reducible Fe(III)) was then recorded. In order to determine the quantity of Fe present in µM, calibration curves were obtained with standards using $FeSO_4$.

6.3.7 Light Microscopy

Meteorite samples from experimental flasks and controls were examined under light microscopy using the Leica DMRP microscope equipped with epifluorescence. Meteorite samples were mounted on glass slides and viewed under 40x magnification. A solution of Syto-9 (See Chapter 3) was introduced to the surface of meteorite samples in an attempt to visualize cells associated with meteoritic materials.

6.3.8 SEM

Scanning Electron Microscopy (SEM) was used in addition to epifluorescence microscopy to examine the surfaces of meteorite samples. Abiotically and biotically altered meteorite

samples were dried in a desiccator and attached to aluminium stubs. These samples were viewed by SEM at 20 kV accelerating voltage and 7-15 mm working distance. The SEM used was housed in the Planetary and Space Sciences Research Institute at the Open University, and was a Quanta 3D dual beam FIB SEM (FEI, Oregon, USA). The meteorites were sufficiently conducting to prevent charging, and carbon coating was therefore not required for use of the SEM. Energy Dispersive Spectroscopy (EDS) was carried out with a count time of 50-100 s. Data analysis was performed with the software, Inca (Oxford Scientific Instruments, Oxford, UK) to study the composition of the samples. Additional SEM images were acquired at the University of Tübingen using a LEO Model 1450 VP (Variable Pressure) with either Everhart-Thornley SE-detector or 4-quadrant BSE-detector (LEO Electron Microscope Ltd., UK).

6.4 RESULTS

When iron meteorites were introduced to the culture medium, a rapid release of reduced iron occurred in the first day. In biotic experiments, this reduced iron was oxidized by the organisms over a period of 15-20 days. In abiotic experiments, a lack of biotic iron oxidation meant that levels of reduced iron liberated from the meteorite in the acidic medium remained high. The comparison between these abiotic and biotic levels of oxidized iron can be seen in **Figure 99**. The quantity of iron was far higher (roughly 50 times greater) in experiments using $FeSO_4$ as a source of iron.
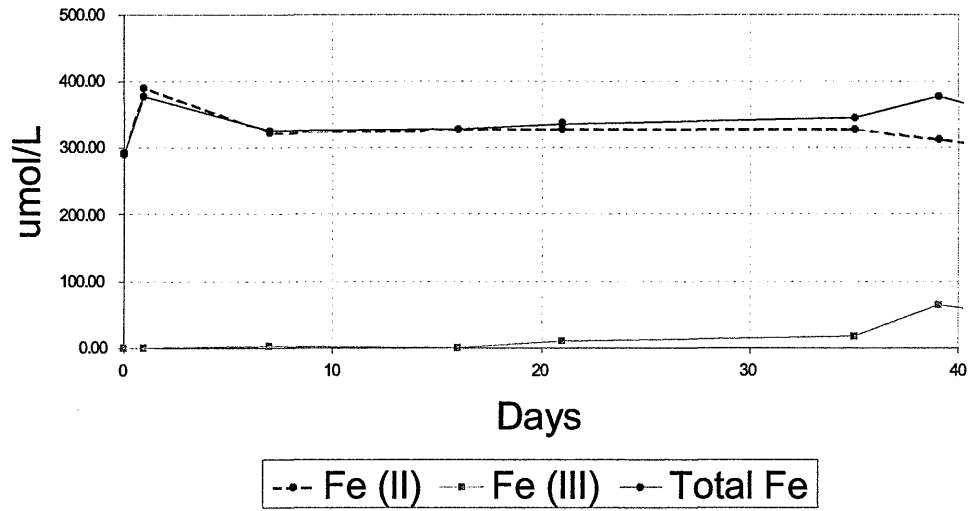
Sol A + Meteorite
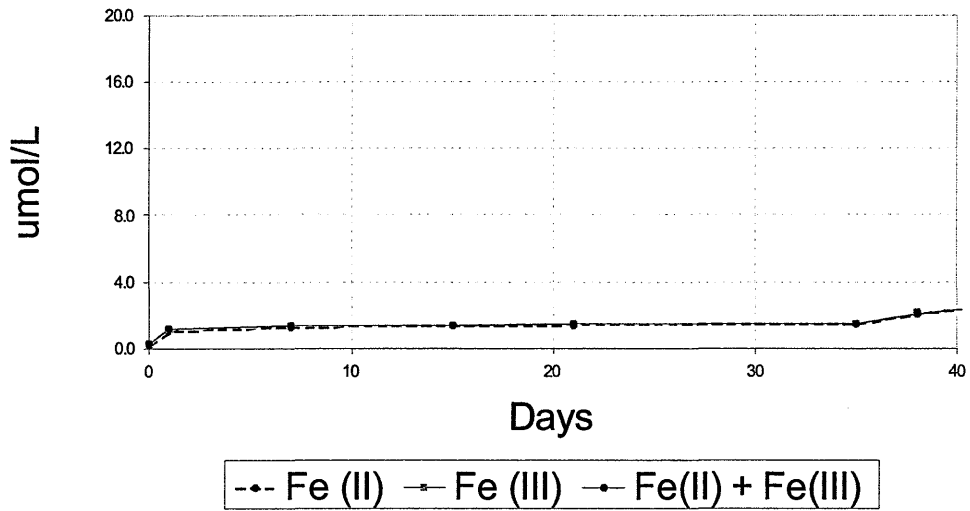
Sol A + Thiobacillus

Sol A + Sol B + Thiobacillus

A4 Sol A + Thiobacillus + Meteorite

Fe (II) Fe (III) Total Fe   Days
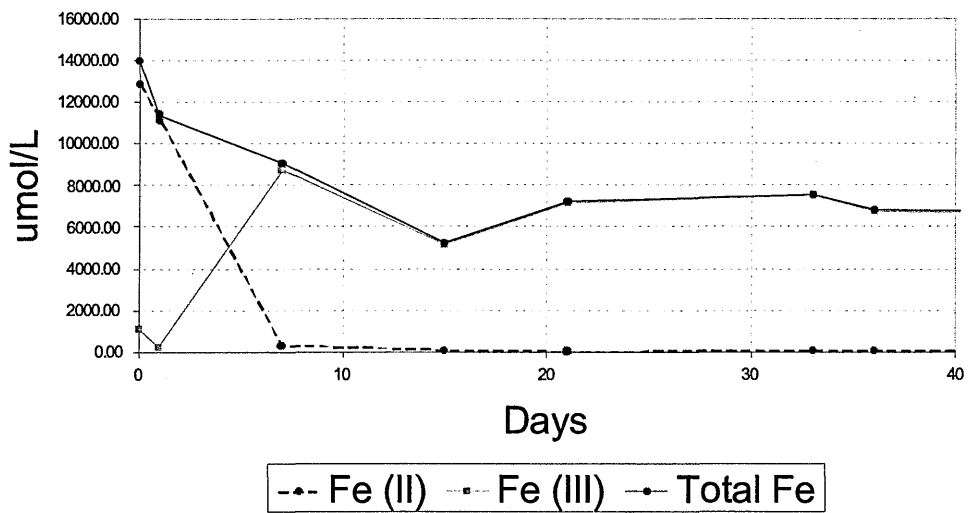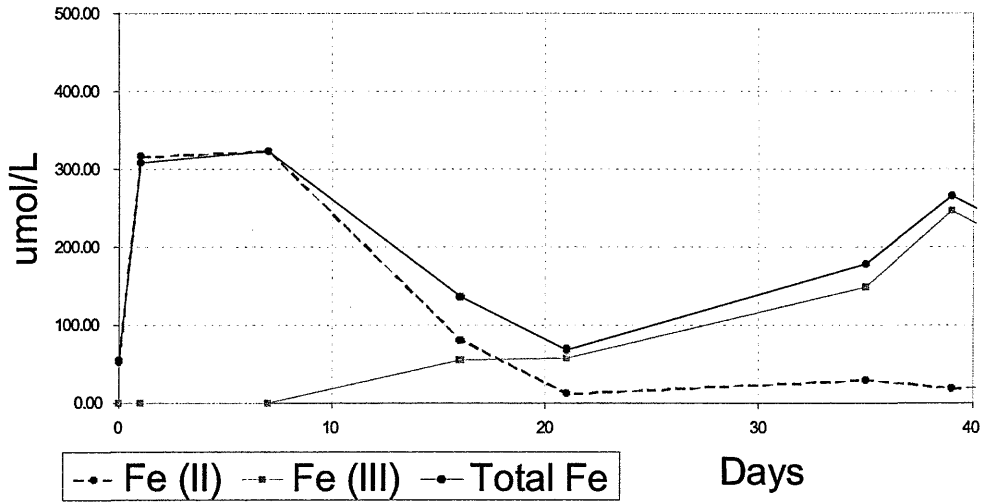
B1 Sol A + Meteorite

Fe (II) Fe (III) Total Fe
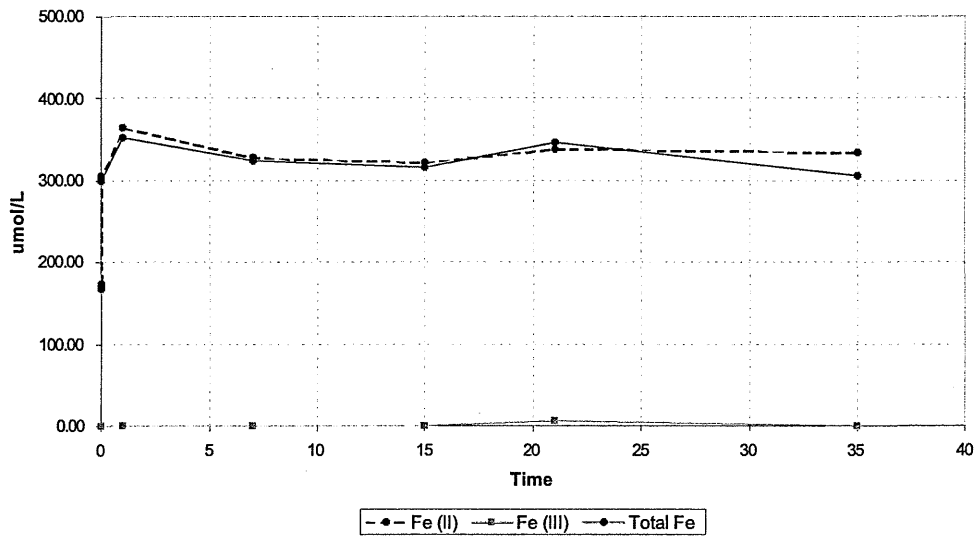
B2 Sol A + Thiobacillus

Days

Fe (II) Fe (III) Total Fe
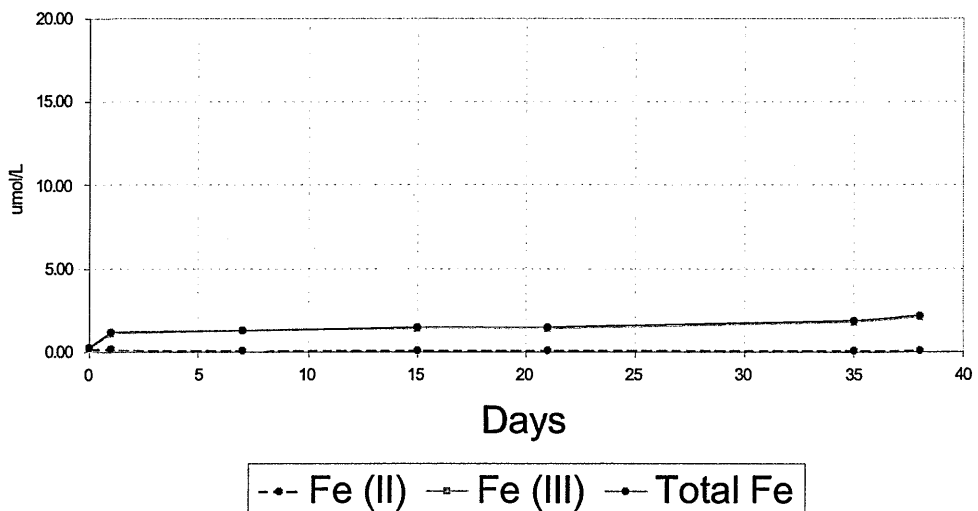
Sol A + Sol B + Thiobacillus
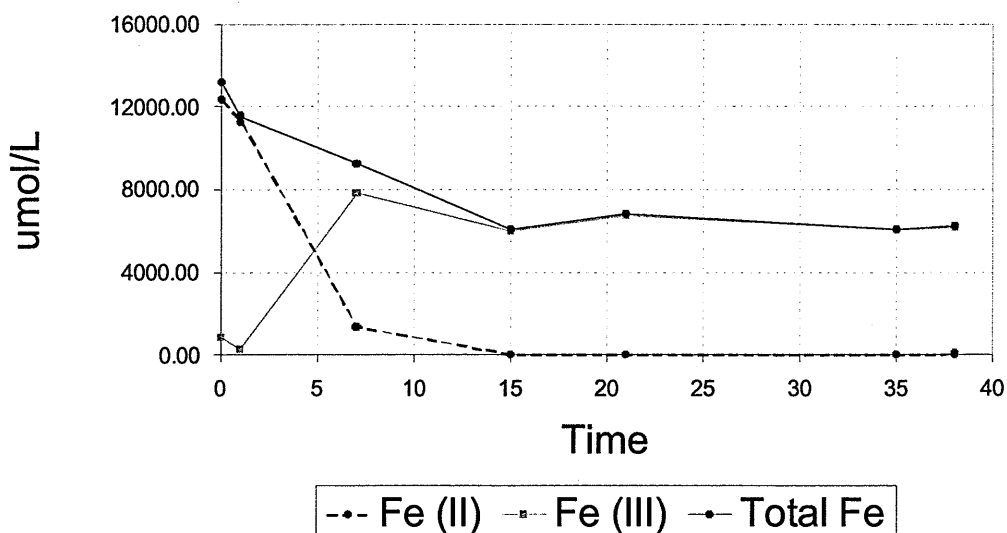
Sol A + Thiobacillus + Meteorite
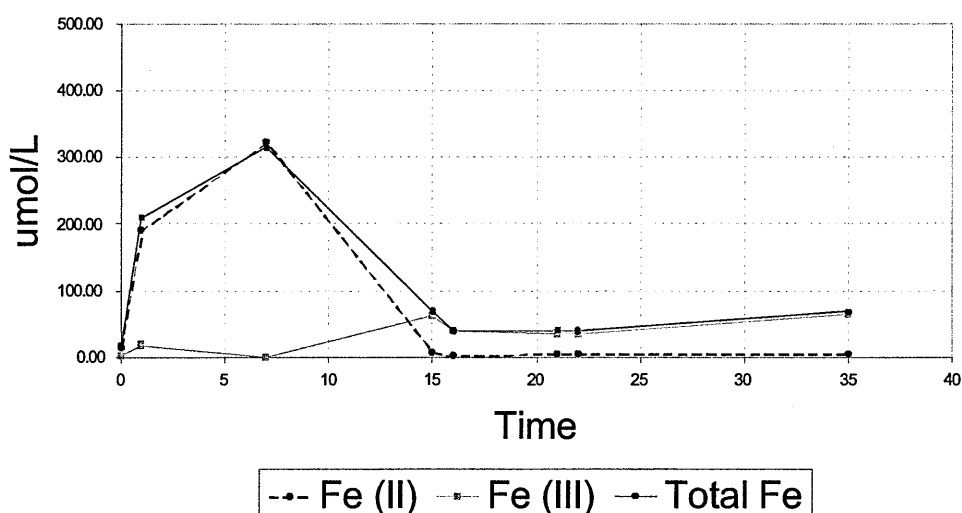
Figure 99 - Two representative experiments (left and right) showing oxidation of iron released from Casas Grandes meteorite by A. ferrooxidans. A1 and B1, solution A and meteorite without organisms; A2 and B2, solution A and organisms without meteorite (iron source); A3 and B3, control experiment with solution A, B (FeSO4) and organisms; A4 and B4, experiment with solution A, meteorite and organisms.

This was expected, as large quantities of $FeSO_4$ are directly added in this standard culture medium for optimal growth of cultures. A minute quantity of reducible iron, on the order of <4 μmol/L, was detected in the control with Solution A and organisms but no source of iron (i.e. no meteoritic material and no added $FeSO_4$). This small amount of reducible iron is attributed to iron released from organisms inoculated into the medium (either as intracellular iron or minerals attached to the cells). No Fe(II) was detected in this control.

Over the course of the incubation period, total iron measured in these experiments was reduced to roughly a third of the initial soluble iron after 20 days. This 'loss' of iron is attributed to the formation of oxyhydroxides. These could be seen as sheets of orange/red precipitates on the sides of flasks (**Figure 100**). Because this iron had precipitated out of solution, it was not included in aliquots removed for the ferrozine assay. The results for the Cape York meteorite are not shown, but were qualitatively identical to results obtained for Casas Grandes.

Figure 100 - Formation of precipitates during incubations period. (1) Solution A + Meteorite + *T. ferrooxidans.* **(2) Solution A + Meteorite. (3) Solution A + Solution B (4) Solution A + Solution B +** *T. ferrooxidans.* **(5) Solution A +** *T. ferrooxidans.*

The initial cell numbers in the control biological experiment using the standard culture medium (i.e. $FeSO_4$ as an iron source) and the meteorite as an iron source in the presence of organisms ($1.01\pm1.34 \times 10^7$ cells/mL and $9.80\pm0.23 \times 10^6$ cells/mL respectively) increased to $5.05\pm0.90 \times 10^7$ and $5.43\pm0.40 \times 10^7$ cells/mL after 20 days. This showed that Fe(II) from the meteorite did indeed support growth. It must be noted, however, that direct cell enumeration under the microscope was difficult due to the presence of particulate matter and iron precipitates that shielded organisms from view.

Counting became increasingly difficult at late stages of incubation as the amount of precipitates continued to increase.

6.4.1 Schreibersite experiment

Experiments showed growth of *T. ferrooxidans* in the presence of FeP_3 (Bottle 1) based on both cell counts and ferrozine tests. Additionally, growth was indicated in the standard biological control (Bottle 3). This is reflected in the visual appearance of bottles, where a strong orange coloration due to precipitated iron compounds can be seen in the experimental bottle (Bottle 1) and the biotic control in standard culture media (Bottle 3) after 8 days of incubation (**Figure 101**). The abiotic control (Bottle 2) appears clear with no iron precipitates. A slight coloration is apparent in Bottles 4 and 5, which could be due to a natural precipitation of iron compounds. This could indicate that the dark coloration of Bottles 1 and 3 seen early in the incubation is due to rapid precipitation of iron brought on by biological activity. After 39 days of incubation, all bottles showed some amount of iron precipitation (data not shown).

Figure 101 - Schreibersite experiment. (1) Experimental bottle. (2) Solution B + Solution C. (3) Standard culture media: Solution A + Solution B + *T. ferrooxidans*. (4) Solution A + Solution B. (5) Solution A (phosphorus-free) + Solution B + *T. ferrooxidans*.

Experimental bottles (Bottle 1) showed an increase in cell numbers of ~7x by the completion of the experiment in the presence of the schreibersite analogue FeP_3. Cell numbers in standard culture (Bottle 3) increased by a similar ~6x (**Figure 102**). Cell numbers in the control containing no phosphorus (Bottle 5) showed a reduction of numbers by 0.5-0.6x; however, cells remained present even after the complete incubation period of 40 days.

Figure 102 - Cell numbers in Schreibersite experiments bottles 1, 3 and 5.

Ferrozine tests showed conversion of Fe (II) to Fe (III), indicating microbial

activity, in Bottles 1, 3 and 5, which is consistent with the results of cell counts (**Figure**

103). A reduction in total Fe was seen in all bottles as a result of the formation of iron

precipitates that began to collect on the walls of the bottles. At the completion of the

experiment, measurements of total Fe and Fe(III) were similar in bottles 1 and 3, indicating

that nearly all iron in solution had been converted to Fe(III). In bottle 5, only roughly half

of the total Fe had been converted to Fe (III) (**Figure 103**).

Figure 103 - Ferrozine results of schreibersite experiment bottles 1, 3 and 5.

6.4.2 SEM

The milling procedure used to obtain meteorite fragments from the original meteorite produced surfaces that were not uniformly flat, as evidenced by the control meteoritic material (data not shown, see Gronstal *et al.*, 2009 (*In Press*)). For the abiotic control, where meteoritic material was exposed to the growth medium and no organisms were present, the surface of the material was covered by aggregates of near-spherical structures roughly 4-6 μm in diameter, and containing Fe, P and S (as determined by EDS) (data not shown, see Gronstal *et al.*, 2009 (*In Press*)). In the biotic experiment, when *T. ferrooxidans* was present, the surface of the meteorite became covered in both near-spherical mineral aggregates and spherical mineral aggregates covered by ~2 μm long needle-like crystals (data not shown). There did not appear to be any direct association with the crystals and organisms themselves, other than the fact crystals only appeared in biotic experiments.

6.4.3 Epifluorescence Microscopy

When meteorite samples from the experiments were stained with the DNA-binding dye Syto-9, meteorite fragments from experiments containing bacterial cells of *T. ferrooxidans* appeared to show distinct areas of fluorescence (**Figure 104**). This indicates that bacterial cells may have been attached directly to the meteorite surface. However, these fluorescing areas were only viewed on a small portion of the meteorite surface. In experiments containing meteorite fragments without bacterial cells present, no fluorescence could be seen on the meteorite surface (**Figure 104**).
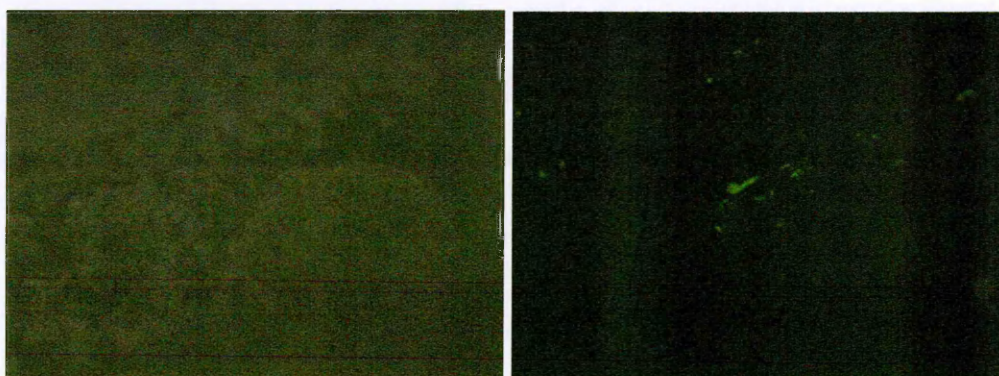
Figure 104 - Meteorite samples stained with Syto-9 from experiments where bacterial cells were present (right) and experiments where no bacteria were present (left). These images were captured at 400x magnification.

6.5 DISCUSSION

It has long been recognized that meteorites can be oxidized in terrestrial conditions to form coatings of iron oxides (Shannon 1927). The connections between oxidation of meteorites and biological activity has more recently become of a topic of interest, due to theories about the role of asteroids, meteors and comets in the origin of life on Earth. Meteoritic material may provide another potential connection between life on Earth and materials originating from space that arrive at the Earth's surface. Exogenous materials can have powerful physical effects when they impact the surface of the Earth – events that, as evidenced by work on the CBIS, can have widespread impact on the habitats of terrestrial microorganisms. If physical remnants of impact remain, these materials can potentially provide a source of half reactions and nutrients for microbial life. On the present day Earth, there is an excess of minerals and nutrients to provide for growth of microbial communities. However, the ability of microorganisms to access meteoritic material demonstrates that such materials may have played a larger role in supporting or aiding life during periods when the environment of the Earth was less hospitable. Extraterrestrial input may have been particularly important on the early Earth when the impact flux was far higher than today. Exogenous materials may also provide an important input of materials on other planetary systems, such as early Mars. To study the relationship between microbial growth and meteoritic material, we examined the ability of iron-oxidising microorganisms to access iron from meteoritic sources, including both iron meteorites and carbonaceous chondrites.

The experiments showed that organisms can indeed play an active role in iron oxidation of meteoritic materials. This action may have important implications in biological mineral weathering. Additional tests showed this to be the case in both aerobic and anaerobic conditions and in neutral and acidic solutions (results not shown, see Gronstal *et al.*, 2009 (*In Press*)).

The breakdown of meteoritic material after its arrival on Earth is likely related to the conditions of the environment in which it lands (Lee and Bland 2003, Bland 2006). However, our studies on the interactions between microbes and meteoritic material indicate that the role of microbiological weathering is also worth consideration. When microbial cultures establish themselves on meteorites, biological oxidation of iron can be rapid in both iron meteorites and carbonaceous chondrites.

The initial soluble iron content in standard culture medium for *T. ferrooxidans* was 50 times higher than in the experiment containing meteoritic material as an iron source. However, even though standard culture medium had such a large amount of soluble iron, complete oxidation of this iron source occurred after ~7 days. In contrast, when meteoritic material was used as a source of iron, complete oxidation of soluble iron took 15-20 days. This indicates a slow release of Fe(II) as meteorites react with the acidic medium. This reaction is evidenced by the formation of gas bubbles at the surface of the meteorite the moment it is introduced into the medium (**Figure 105**). This release of iron likely occurs through the pathway:

Fe (solid) + H_2SO_4 → H_2 (gas) + $FeSO_4$ (aqueous)
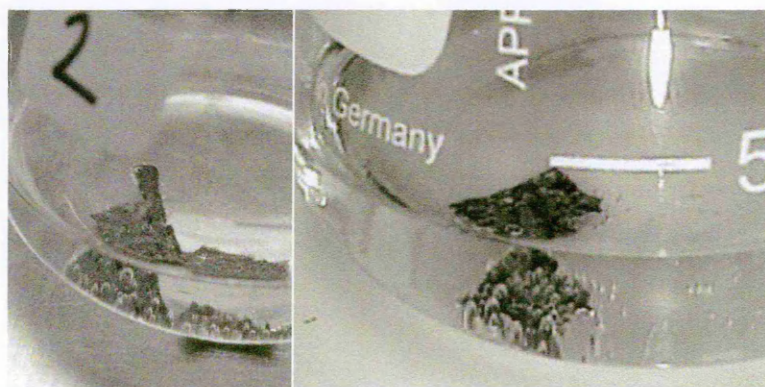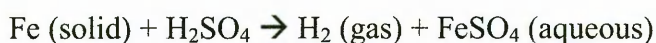
Figure 105 - Bubbles forming when meteorite fragments are added to acidic culture media, indicating the release of hydrogen gas.

As the surface of the meteorite becomes oxidized, release of Fe(II) would be slowed. A similar effect was reported by Bland (1998, 2006) on chondrites.

When meteorites were viewed under various types of microscopy, there was indication that microbial cells could be directly associated with meteoritic material. Styo-9 staining of iron meteorite surfaces did not show a conclusive presence of cells over the surface of iron meteorites. Instead, cell density appeared to be more concentrated in very specific areas, often in linear patterns. It is possible that cells were attaching to areas of the surface where the meteorite was fractured or cracked during processing.

Iron oxidizing microorganisms are found in a variety of environments (Straub et al. 1996, 2004; Emerson and Moyer 1997, 2002; Weiss et al. 2003; Kappler et al. 2005), but their relationship to iron corrosion has not been previously examined. Further studies could help elucidate the potential role of iron oxidizers in this process. Additionally, our study has suggested the possibility of an iron cycle existing within iron-rich meteorites (and iron rich metals), where iron oxidizers and iron reducers could interact to shift iron through redox states, ultimately producing energy from meteorite-derived material to support a microbial community (See Gronstal *et al.*, 2009 (*In Press*)).

6.5.1 Schreibersite experiments

Cultures of *T. ferrooxidans* showed activity in both cell counts and in ferrozine tests when phosphorus in the growth media was replaced with the Schreibersite analogue, FeP_3. However, cultures also survived throughout the length of the incubation period when all phosphorus was removed from the media as indicated by the presence of cells in final cell counts and the conversion of $Fe(II)$ to $Fe(III)$ in cultures. Even phosphorus-starved cells of *T. ferrooxidans* appear to be able to survive for extended periods of time without an input of phosphorus, which is consistent with previous studies. Growth of phosphorus-starved *T. ferrooxidans* has previously been shown to be reduced compared to standard cultures (Jerez et al. 2006; Seeger and Jerez 2006; Varela et al. 1998). When starved of phosphorus *T. ferrooxidans* begins an excessive production of several proteins (Varela et al. 1998), the purposes of which have not yet been determined. Additionally, starvation can cause filamentation of cells (Seeger and Jerez 2006). However, a strain similar to the one we

examined, *Acidithiobacillus ferrooxidans*, was shown to survive in phosphate-limiting conditions by producing a chemical that is able to cleave the chemically inert (and biologically resistant) C-P bond in phosphonates (Vera et. al 2008).

Cell numbers increased (roughly ~6x) in both standard cultures media and in experimental bottles where phosphorus was replaced with FeP_3. Cell numbers in the phosphorus-free control were reduced by roughly half at the end of the experiment. This is consistent with reduced growth rates observed previously in phosphorus-starved cells of *T. ferrooxidans* (Jerez et al. 2006; Seeger and Jerez 2006; Varela et al. 1998). This indicates that FeP_3 was indeed providing a source of phosphorus in the experimental bottle, where increases in cell numbers and conversion of Fe(II) to Fe(III) was similar to the standard culture control. The implication of this experiment is that meteorites can feasibly provide additional nutrients such as phosphorus to support microorganisms in nutrient-depleted systems if meteoritic material is exposed to microorganisms after delivery into the environment.

6.5.2 Implications

The experiments on the survival of *T. ferrooxidans* using meteoritic material as a source of iron have shown that meteorites can supply inputs for microbial growth. This was shown by increases in cell numbers, particularly in experiments using iron meteorites. González-Toril et al. (2005) have previously reported that acidophilic iron oxidisers can use iron meteorites as a source of reduced iron. However, contrary to their conclusions, the reduced iron is primarily provided by the acidic conversion of metallic Fe to Fe^{2+} rather than the organisms directly using metallic Fe from the meteorite.

Recently, it has been suggested that Fe(II) is the first element to become limiting to potential chemolithoautotrophic life on Mars according to potential nutrients and redox couples in Martian soil (Jepsen *et al.* 2007). If exogenous material could serve as an input of Fe(II) into such systems, this limitation could be mitigated. The theoretical data from Jepsen *et al.* (2007) highlights the importance of available Fe(II) in biological systems and,

when considered with our experimental data, local limitations to Fe(II) could potentially be overcome by delivery of extraterrestrial material in aerobic and anaerobic (see Gronstal *et al.* 2009 (*In Press*)) environments.

6.5.3 Experimental Applications

The experiments performed on the ability of microorganisms to access resources in meteoritic material may also have important implications for the future of human space exploration. Asteroids are known to harbour a number of useful elements, including platinum, and could serve as an important source for in situ resource utilization for human space settlements and terrestrial industry (Kryzanowski and Mardon 1990; Sonter 1997; Busch 2004). Today, microorganisms are already playing an important role in extracting resources like copper and gold through a process using acidophilic iron oxidizers in a technique dubbed 'biomining' (Norris et al. 2000; Stott et al. 2003). Iron oxidizers provide a source of Fe(III), which subsequently causes oxidation of sulphidic minerals (Clark and Norris 1996; McGuire et al. 2001) and production of sulphuric acid that aids in breaking apart the rock to facilitate extraction of minerals (e.g., Pronk and Johnson 1992; Schroeter and Sand 1993; Solisio et al. 2002; Rawlings 2005). To develop this process for extraterrestrial material, the first step is to ensure that iron-oxidizing microorganisms can oxidize reduced iron without suffering any toxic affects from the parent material. In this study, we have shown that *T. ferrooxidans* is able to oxidize reduced iron originating from carbonaceous chondrites and iron meteorites. These materials also contain sulphides, which could act to sustain leaching reactions similar to the process described above. This indicates that, in the future, asteroidal material could be exposed to organisms in oxygen pressurized batch reactors to aid in biomining of important resources for long-duration human space exploration.

Chapter 7: Conclusions Drawn from the 2005 ICDP-USGS Deep Drilling of the CBIS and Discussion of Future Work

The 2005 ICDP-USGS deep drilling of the CBIS was the first large-scale drilling effort of its kind to retrieve a contiguous core throughout the depth of an impact structure (**Chapter 1**). During retrieval of core materials, techniques for assessing the level of biological and fluid contamination in cores were implemented (**Chapter 2**). For the first time, microbiology and molecular biology techniques could be used to examine microbial communities in the deep subsurface associated with an impact structure.

The CBIS represents one of the best-preserved shallow-marine impact sites known on Earth. The unique set of conditions under which the structure formed left behind geological traces deep below the surface that still have a profound effect on subsurface geology and hydrology of the region (see **Chapter 1**). Prior to drilling, it was thought that these lasting effects on the subsurface environment would influence the re-colonization of subsurface materials by microbes after impact target materials cooled and reached a temperature suitable for microbial communities to thrive. The potential isolation of impact-altered environments within the crater over geologic timescales could also provide an environment containing novel species of microorganisms that are distinct from those in surrounding subsurface environments outside of the crater structure.

The first step in examining the microbial communities present in the CBIS was to obtain core samples suitable for molecular and microbiology studies. Through a strict implementation of contamination assessment procedures, core material retrieved from the CBIS provided the first opportunity to examine microbial communities in the deep subsurface throughout the depth of an impact structure. As expected, implementation of as many contamination assessment techniques as time and funding allows provides the best method for acquiring clean material. The use of multiple techniques also ensures that if one

technique fails, others can serve to replace it, meaning that a failure in a single technique (i.e. a failure in delivery of microspheres for example) does not compromise an entire section of core.

Four distinct contamination assessment techniques were implemented in the CBIS study, including fluorescent microspheres, perfluorocarbon tracers, analysis of DOC content in porewaters and analysis of acid polar components in porewaters. Additionally, clone libraries were constructed from drilling fluids, providing a list of potential contaminant organisms present in fluids that could then be cross-checked with organisms obtained in culturing studies. Cores for molecular and microbiology studies were collected at regular intervals, providing a contiguous core from which direct enumeration of cell numbers, culturing of native organisms and molecular analysis could be performed.

7.1 Microbial Communities in the CBIS

Large-scale impact events on Earth may be relatively rare today, but it is thought that our planet suffered frequent and violent impacts early in its history. Many periods of high impact rates are thought to have occurred during and after the origin of life. Large impacts can have devastating repercussions for life at the surface of the Earth, where the energy released by the impact can cause local, and sometimes global, changes in the environment. Studies have identified potential links between impacts and periods of mass extinction and global climate change. The affects of impact events on the subsurface environment, however, are little known.

Following successful collection of CBIS cores, direct enumeration of microbial cell numbers were performed on material drawn from the interior region of the cores. Enumerations were performed every 12 m on average, with the shortest distance between samples being 1.74 m and the largest being 31.09 m. After testing a selection of DNA-binding fluorescent dyes and staining techniques, the final method for enumerations selected involved the use of 4', 6-diamidino-2-phenylindole (DAPI). Additional enumerations were performed on core samples that showed a high level of background

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fluorescence using a cross-staining method with DAPI and an additional dye, Sybr Green I. Direct enumerations were performed throughout the entire length of the CBIS core, providing a map of general cell numbers from the top to the base of the structure.

Cell enumeration estimates revealed a pattern of microbial abundance corresponding to the lithological transitions within the crater - as defined by the geology team of the 2005 ICDP-USGS Deep Drilling program. The results of enumeration studies revealed three general microbiological Zones of the CBIS core. The first Zone showed a logarithmic decline in cell numbers in the upper regions of the core, and is consistent with trends in other deep subsurface environments, both terrestrial and in marine sediments. However, the gradient of decline seen in the CBIS was more pronounced, particularly when compared to studies in deep sea marine sediments.

The second microbiological Zone of the CBIS core appeared to be biologically deprived, with most cell numbers falling below the detectable limit for all enumeration methods attempted. This indicates that this section of the core is impoverished and contains materials that do not provide a suitable habitat for thriving microbial communities.

The third microbiological Zone of the CBIS corresponds to an increase in total cell numbers in the deepest section of the core. This increase indicates that some form of post-impact recolonisation has occurred. Microbial communities at this depth would not have been able to survive the intense thermal pulse generated by the impact, and recolonisation would have occurred after sediments had cooled to a temperature suitable for life to survive. The process of recolonisation was likely facilitated by fracturing of target rocks, with the cracks and fissures providing new spaces for colonies to inhabit.

In addition to enumerating the total microbial cell numbers throughout the length of the CBIS, core sediment samples were also examined for the presence of living, cultureable organisms. Obtaining live cultures from sediments provides a means of identifying specific species types present in the subsurface environment. Enrichment cultures were obtained from multiple depths in the CBIS core and using varied culture

media types. Successful cultures corresponded to microbiological Zones 1 and 3, where microbial cells were above the detectable limit in enumeration studies. No successful cultures were obtained from microbiological Zone 2. These results support the results of enumeration studies and the idea that microbiological Zone 2 is a biologically impoverished region where the numbers of cultureable microorganisms are low.

The most successful media for returning microbial cultures was found to be a media for general heterotrophic anaerobic organisms. However, additional cultures were obtained with media for iron reducers, sulfate reducers, iron oxidizers and humic acid utilisers. The depths from which these organisms were obtained could often be related to changes in porewater chemistry. This indicates the presence of diverse metabolic processes within the microbial community of the CBIS. However, many of these organisms are difficult to culture under laboratory conditions, and the cultures that were obtained were often short-lived and presented great difficulty in maintaining healthy cells through multiple inoculations. Additionally, many of the enrichment cultures appeared to be composed of only single cell-types, indicating that the enrichments could be dominated by a single species.

The most robust cultures obtained in enrichment studies were then used to obtain isolate cultures of single species. Isolate cultures provide a means of studying the physiological characteristics of specific members in the general microbial community. Isolates were obtained from depths within microbiological Zones 1 and 3, as defined by enumeration studies. As with the enrichment cultures, isolate cultures proved extremely difficult to maintain under laboratory conditions. Growth of cultures was difficult across multiple inoculations, and many cultures died after only short periods of incubation. This caused additional limitations in obtaining cultures that were robust enough for physiological studies. Attempting to determine optimal growth conditions for isolates and identification of specific species through molecular biology studies was hindered by the

fastidious nature of cultures. Extracting amplifiable DNA from these cultures also proved difficult, and much of the DNA that was amplified was un-sequenceable.

Attempts to obtain both enrichment and isolate cultures from the CBIS highlights the fact that the majority of microorganisms present in the deep subsurface environment are likely uncultureable using standard laboratory techniques. The apparently low diversity in enrichment cultures, which often appeared to be composed of a single cell type, also supports this idea. When DNA was extracted from enrichments and sequenced, the sequence produced was often clean with very few un-interpretable base pairs. This is further indication that enrichment cultures may have been composed of single species types, or that a single species was highly dominant in the enrichment so that DNA sequences from additional species were present in such low numbers as to not interfere with sequencing.

7.2 Relevance to the Earth

Even though impact processes can have profoundly devastating effects for life at the surface of the Earth, this study shows for the first time that impacts can potentially serve to create new environments for microorganisms in the deep subsurface. Impacts could potentially sterilize subsurface materials by generating extreme compacting forces and thermal heating. However, the force of impact can also serve to fracture deep subsurface rock and 'reshuffle' subsurface materials and nutrients to provide for re-colonization by microorganisms following dissipation of thermal heat.

Once an impact has occurred, it is possible for microorganisms to re-colonize these new environments, as evidenced by both microbial enumeration and culturing studies performed on cores from the 2005 ICDP-USGS deep drilling of the Chesapeake Bay Impact Structure. This may have implications in understanding how impacts affected life during periods of high impact rates on Earth. Although high impact rates could have made the surface of the planet very inhospitable, the deep subsurface of the Earth may have provided a safe haven for microbial life to thrive.

Studies on the ability of microorganisms to access sources of nutrients from meteoritic material provide further evidence that the arrival of extraterrestrial material may aid some microbial communities on Earth. Meteorites were used to supply inputs for growth of the iron oxidizing microorganism, *T. ferrooxidans*. Iron from iron meteorites provided a sole source of Fe (II) to support continuous growth of *T. ferrooxidans* cultures. Additionally, FeP_3 was used as an analogue for the meteoritic component Schreibersite to show that meteorites could potentially provide a source of phosphorus to support microbial communities.

The results of these studies together highlight the potential beneficial aspects of impact cratering and the delivery of exogenous materials for life on Earth. Impacts do not purely act as sterilizing events, and may in fact create new environments in the deep subsurface for microbial communities to inhabit.

7.3 Relevance to Habitability in the Deep Subsurface of Other Celestial Bodies

There is no environment present at the surface of planets in our solar system aside from Earth that is known to support life. In fact, it is likely that the conditions present at the surface of our neighbouring celestial bodies are inhospitable for life as we know it. However, the conditions in the subsurface of planets like Mars remain unknown.

Many scientists have hypothesized about the potential for past life on Mars, both above and below the surface (e.g. Boston *et al.* 1992; Klein *et al.*1976; McKay *et al.* 1996; Sleep and Zahnle 1998), and impacts have been cited as a major obstacle for the continued existence of life in the martian environment (Sleep and Zahnle 1998). To survive impacts, life would have to persist deep below the planet's surface (Sleep and Zahnle 1998). The research presented here shows that microorganisms do indeed persist at great depths, and can act to re-colonize impact-altered materials in these deep subsurface environments. As is the case with the Earth, this study shows that if microbial life was ever present in the

deep subsurface of Mars (or any other rocky planet), impact events would not necessarily be detrimental to these communities.

The ability of microorganisms to access nutrients and minerals present in meteoritic material, as shown by work presented here, also indicates that exogenous material could provide inputs for life on planetary bodies that are deprived of the necessary components for microbial growth. In martian soil, for instance, Fe (II) may be a limiting element for chemolithoautotrophic life (Jepsen *et al.* 2007). As proven with work on *T. ferrooxidans*, this limited availability of Fe (II) could be overcome in some locations with the delivery of iron-rich meteorites.

7.4 *Potential for Future Studies*

Samples for biological studies were collected from the CBIS in 2005. Over time, the integrity of these samples becomes reduced in terms of their usefulness in further experiments. When samples are stored for extended periods of time, the microbial community present in the samples can change. Many organisms will eventually die off as nutrients in the sample become depleted. Organisms better suited to survival in the conditions that the samples are stored in can outgrow less competitive species, altering the community structure of the organisms present. The likelihood of obtaining cultureable organisms from CBIS samples many years after collection is greatly reduced.

When cells die off they leave behind biological traces of their presence, such as DNA and RNA molecules. Samples collected from the CBIS may still prove useful for DNA extraction and analysis in order to identify the potential presence of further organisms in the subsurface environment. It is not certain how long DNA can persist outside of the cell in soil environments (Blum *et al.* 1997), but any amplifiable DNA that can be obtained could be useful in providing further information about communities of microorganisms in the CBIS. Additional analysis on biomolecules, such as RNA, rhibosomes and amino acids, could prove useful for similar reasons (Elsas *et al.* 2006, p341; Felske *et al.* 1996). These studies would be of particular interest in samples that have

been in cold storage at the USGS in Reston, Virginia, and have not been manipulated since the time of collection. Frozen samples will not contain the exact makeup of biomolecules present in the natural environment due to disruption or destruction of cells and biomolecules during the freezing and thawing process (Elsas *et al.* 2006, p. 341). Yet, because the microbial environment of the CBIS is unknown, any further information about potential members of the microbial community would be of use.

The usefulness of sediment samples for enumeration stored in formaldehyde solution is also questionable, as cells present in the sediments will naturally degrade over time in spite of being fixed in solution. Cells fixed with formaldehyde do suffer some amount of distortion and many structures in the cell are damaged (Fox *et al.* 1985). Studies have shown that fixed samples remain useful with stains such as DAPI for extended periods of time; however no studies have compared cell counts from samples after multiple years of storage (e.g. Porter and Feig 1980). Although it would take considerable effort, a study comparing the changes in total cell numbers present in samples after more than three years of storage to counts obtained shortly after sample collection could be of interest.

The nature in which formaldehyde solutions interact with cellular components (Fox *et al.* 1985) makes analysis of biomolecules like DNA in fixed samples difficult. Attempts to extract amplifiable DNA from samples fixed in formaldehyde were attempted in the laboratory at the Open University. However, even after extensive washing to remove formaldehyde, none of these extractions produced amplifiable DNA.

Possibly the most important set of further studies to be performed on samples from the CBIS is continued examination of the cultureable organisms obtained. Although these cultures are difficult to maintain, further DNA extractions need to be performed on a number of samples in order to obtain sequenceable DNA. Additionally, determining better growth conditions for these organisms in order to obtain higher densities of cells in culture would aid in obtaining materials for G+C analysis and additional growth studies to identify specific species present in the CBIS.

The USGS research program surrounding the Chesapeake Bay Impact Structure is a continuing project. Although future deep drilling efforts similar to the 2005 ICDP-USGS project are not currently in preparation, small-scale drilling projects at other sites within the crater structure could be undertaken in the future. With the data provided in this thesis, it would be of great benefit to implement contamination control procedures to include biology in the suite of studies used to examine future cores. This would provide additional information concerning the effects of cratering on the microbiology of the subsurface in a site where an initial set of data now exists. The primary cost involved with acquiring drilling samples is in the drilling equipment and procedure itself, and it would seem that the additional cost of including contamination control procedures is worth the potential knowledge gained by including an entire field of additional scientific study.

Ideally, additional cores from outside of the impact crater would be of great use in determining what the microbial environment in the deep subsurface of the Atlantic Coastal Plain would have looked like prior to the impact event. Cores have been dug previously in sites near the crater (e.g. Lehman *et al.* 1995; McKinley and Colwell 1996); but none of these represent contiguous cores of similar depth to that of 2005 ICDP-USGS drilling of the CBIS, or with sufficient contamination controls in place for biological studies to be implemented.

7.4.1 Studies Beyond the CBIS

Crater sites exist all over the world, and many are the subject of drilling efforts (e.g. Kershaw *et al.* 2007; Koeberl *et al.* 2007; Sharpton *et al.* 1996; Sturkell 1998; Sturkell *et al.* 1998; Urrutia-Fucugauchi *et al.* 1996; von Dalwigk and Ormo 2001). However, biological studies have not yet been included in drilling projects at previous crater sites. As the CBIS is only one example of an impact-altered subsurface, it would be of great interest to obtain biologically useful core samples from other craters on Earth that occurred in a range of environments. By expanding the sample set of craters, more accurate inferences could be made about the general affects of impact events on subsurface microbial habitats.

7.5 Summary

Impact events have been viewed as devastating natural occurrences for life on Earth. Impacts have been cited as a contributing factor in mass extinctions and a potential obstructing force for the origin and development of life. The work presented here shows that this is not necessarily the case. In fact, some of the effects of impact events may actually be beneficial for life in the deep subsurface of Earth. Impacts can generate new habitat by redistributing nutrients in the subsurface and by generating fractures and pore spaces in which microorganisms can re-colonize. Exogenous material delivered to the planet may also provide important nutrients and minerals in otherwise impoverished locations. Impacts could also present the same beneficial consequences for the habitability of subsurface environments on other rocky celestial bodies. Studies on the microbial environment in the deep subsurface of the Chesapeake Bay Impact Crater provide reason to re-examine the role of impact events in the development of life and continual habitability of Earth.

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Appendix

Appendix I – Safety Information of DNA-binding Dyes

Table 41 Important safety information in use of selected epifluorescent stains used in direct enumeration and/or visualization of microbial cells in CBIS core samples and cultures.

Stain	Source of MSDS	Noted issues
Acridine Orange	MP Biomedicals, LLC. (2005)	• toxic to humans • potentially harmful if exposed through inhalation, ingestion or contact with skin and eyes • Avoid chronic, local and specific exposures
DAPI	KPL, Inc. (2006)	• irritant • may emit toxic fumes if exposed to flame • harmful if swallowed, inhaled or absorbed through the skin • avoid inhalation, ingestion, skin and eye contact
SYTO-9	Invitrogen Corp. (2008)	• irritant (skin and eyes) • harmful if swallowed, inhaled or absorbed through skin • potentially harmful, avoid exposure • Listed on the US Toxic Substances Control Act (TSCA)
SYBR Green I	Roche Diagnostics (2006)	• Irritant (eyes) • Harmful if swallowed or absorbed through skin or eyes

Appendix II – Culture media recipes

1. Heterotrophic Media DH (Het Med DH)

Derived from D'Hondt *et al.* 2003. Explanatory Notes 2003, Proceedings of the Ocean Drilling Program, Volume 201; also described by Sub *et al.* FEMS Microbiology Ecology, 51, 109-121.

Made in 1 Litre

24.32 g NaCl (or altered according to depth)
10g $MgCl_2$
1.5 g $CaCl_2$
0.66 g KCl
4.0 g Na_2SO_4
1mL KBr (0.84M)   (or use 100mg)
1mL H_3BO_3 (0.4M)   (or use 24mg)
1mL $SrCl_2$ (0.15M)   (or use 40mg)
1mL NH_4Cl (0.4M)   (or use 20mg)
1mL KH_2PO_4 (0.04M) (or use 5mg)

Autoclave/cool then add

30mL of 1M $NaHCO_3$
With 1mL selenate/tungstate
1mL vitamin solution
1mL trace element solution
0.140g $FeSO_4$
10 mL of Na_2S (0.2M)   [100ml stock solution with 4.8g in 100mL]

2. Heterotrophic Media 1 (Het Med 1)

Adapted from: Stevens, TO. 1995 Journal of Microbiological Methods, 21, 293-303

General medium (made in 1 litre H_2O)

First Add:
0.25 g	KH_2PO_4
0.35 g	K_2HPO_4
0.015 g	$CaCl_2 . 2H_2O$
0.02 g	$MgCl_2 . 6H_2O$
0.007 g	$FeSO_4 . 7H_2O$
0.005 g	Na_2SO_4
0.5 g	NH_4Cl
1 g	glucose
1 mL	Trace element solution (Trace Metals)

Add NaCl as appropriate (24.32 g or adjusted according to depth of sample)

Adjust to pH 7.5

Then Add:
0.05 g	peptone
0.05 g	typtone
0.1 g	yeast extract
0.02 g	Na-acetate
1 g	casamino acids (Tsai et al. 1995)
1 mg	resazurin (Tsai et al. 1995, AEM, 45, 301-307)(1 mL of 0.1%)

Autoclave

Add:
1 mL	vitamin solution (see below)
10 mL	Na_2S (0.2M)
1 mL	selenite/tungstate solution

Vitamin Solution:
Made to 100mL
Add:
2 mg	biotin
2 mg	folic acid
10 mg	B6 (pyroxidine)
10 mg	riboflavin
10 mg	thiamine
5 mg	pantothenic acid
5 mg	nicotinamide
10 mg	B12
5 mg	PABA
6 mg	lipoic acid

3. Media for anaerobic iron-oxidisers

Per litre:

0.3 g NH4Cl
0.05g MgSO4.7H2O
0.4g MgCl2.6H2O
0.6g KH2PO4
0.1g CaCl2.2H2O

Autoclaved and cooled under N2/CO2(90:10)

Then add

1mL trace element soln.
1 mL selenite-tungsten soln.
30mL sodium carbonate
1mL vitamin soln.
1 ml thiamine soln.
1mL vitamin B12 soln.
7.5 mL sodium sulphide

And then add FeSO4 from a 1M anoxic stock soln to a final concentration of 10mM
And 4mM NaNO3 from a stock soln of 400mM.

Must be at pH 7.0
Per mL of NaS add 0.13mL of 1M H2SO4

Solutions:

Vitamin Solution
100mL sodium phosphate buffer (10 mM; pH 7.1)

Biotin	1 mg
p-Aminobenzoic acid	4 mg
Nicotinic acid	10 mg
D-Ca-pantothenate	5 mg
Pyridoxine-HCl	15 mg

(To make 200mL of 10mM sodium phosphate buffer add 0.33mL of 2M monobasic sodium phosphate to 0.67 mL of 2M dibasic and check pH).

Filter sterilized and kept at 4C in the dark.

Trace metals

NA-EDTA	5.2 g	
FeSO4.7H20	2.1	g
H_3BO_3	30	mg
$MnCl_2$ x 4 H_2O	100	mg
$CoCl_2$ x 6 H_2O	190	mg
$NiCl_2$ x 6 H_2O	24	mg
$CuCl_2$ x 2 H_2O	2	mg

ZnSO$_4$ x 7H20	144	mg
Na$_2$MoO$_4$ x 2 H$_2$O	36	mg
Distilled water	987	ml

Leave 1/3 head space with N2. Autoclave

Selenite-tungstate soln

40 mg NaOH
0.6 mg Na$_2$SeO$_3$ x 5 H$_2$O
0.8 mg Na$_2$WO$_4$ x 2 H$_2$O,
100 mL distilled water
Autoclave with 1/3 head space with N2

Thiamine soln
10 mg of thiamine in 100mL of 25mM sodium phosphate buffer pH 3.4

(To make 1000mL of 25mL phosphate buffer at pH 3.4. Add 11.25 of 2M monobasic sodium phosphate buffer and 1.25 2M dibasic sodium phosphate. Adjust to pH 3.4 with monobasic sodium phosphate)

Filter sterilized

Bicarbonate soln.
84g of NaCO3 in 1L water
Dispense in small bottles with 1/3 head space.
Purge with CO2 with repeated shaking. Then autoclave

Vitamin B12 soln.
5mg cyanocobalamine in 100mL distilled water. Filter sterilized

Sulfide soln. 0.2M
Clear colourless sodium sulphide crystals required. 48 g/L
Dissolve in distilled water under N2
Autoclave under N2

FeSO4
Make 1M anoxic stock soln (under N2 again)

NaNO3
Make 400mM stock soln

To prepare medium.
Prepare basal medium

4. Alternative recipe: Media for anaerobic iron-oxidisers

Per litre:
0.3 g NH4Cl
0.05g MgSO4.7H2O
0.4g MgCl2.6H2O
0.6g KH2PO4

0.1g CaCl2.2H2O

Autoclaved and cooled under N2/CO2(90:10)

Then add:
1mL trace element soln.
1 mL selenite-tungsten soln.
30mL sodium carbonate
1mL vitamin soln.

FeSO4 from a 1M anoxic stock soln to a final concentration of 10mM
and 4mM NaNO3 from a stock soln of 400mM.

For reduction 1mg/L resazurin could be used (Tsai et al. 1995, AEM, 45, 301-307.

Must be at pH 7.0

Vitamin solution: (Stevens, TO. 1995 Journal of Microbiological Methods, 21, 293-303)
2 mg biotin,
2 mg folic acid,
10 mg B6 (pyroxidine),
10 mg riboflavin,
10mg thiamine,
5 mg pantothenic acid,
5 mg nicotinamide,
10mg B12,m
5 mg PABA,
6 mg lipoic acid)

Trace element soln. (from Widdel and Bak, the sulphate reducers)

NA-EDTA	5.2	g
FeSO4.7H20	2.1	g
H_3BO_3	30	mg
$MnCl_2$ x 4 H_2O	100	mg
$CoCl_2$ x 6 H_2O	190	mg
$NiCl_2$ x 6 H_2O	24	mg
$CuCl_2$ x 2 H_2O	2	mg
$ZnSO_4$ x 7H20	144	mg
Na_2MoO_4 x 2 H_2O	36	mg
Distilled water	987	ml

Leave 1/3 head space with N2. Autoclave

5. Iron Reducer Medium (IR Med DH)

Made to 1 Litre

NaCl	30 g
Na_2HPO_4	0.14 g
$CaCl_2.2H_2O$	0.14 g
$MgCl_2.6H_2O$	7.58 g
NH_4Cl	0.25 g
KCl	0.33 g
$Na_2SeO_3.5H_2O$	0.0005 g
$Fe(NH_4)_2(SO_4)_2.6H_2O$	0.01 g
Wolfe's trace mineral solution	10 mL

Yeast extract	1 g	(0.1 % wt/vol)
Peptone	1 g	(0.1 % wt/vol)
Glucose	0.5 g	(0.05 % wt/vol)
Na_2S	0.5 g	(0.05 % wt/vol)
Ferrihydrite	10 g	(1 % wt/vol)
Magnetite	10 g	(1 % wt/vol)
Wolfe's vitamin solution	1mL/L	

6. Sulfate Reducer Media (SR DH)

D'Hondt *et al.* (2003)

Made per Liter, Prepare under N_2

MJ - artificial seawater solution:

NaCl	30 g (or as needed)	
Na_2HPO_4	0.14 g	
$CaCl_2\ 2H_2O$	0.14 g	
$MgCl_2\ 6H_2O$	7.58 g	
NH_4Cl	0.25 g	
KCl	0.33 g	
$Fe(NH_4)_2(SO_4)_2\ 6H_2O$	0.01 g	
Trace mineral Solution	1 mL	
Selenate Tungstate	1 mL	(or $Na_2SeO_3\ 5H_2O$)

Then add:

Sodium lactate	0.05 g
Sodium acetate	0.05 g
Sodium formate	0.05 g
Sodium fumarate	0.05 g
Glucose	0.05 g
Na_2SO_4	0.1 g
Cystein-HCl	0.05

Vitamin solution	1 mL
Sodium Sulfide-Na_2S	10 mL (0.2M)

7. Sulfate Reducer Media (SR Med 2)

Pfennig *et al.* (1998)

Solution 1 - mineral salts base

Na_2SO_4	3.0 g
NaCl	1.2 g
KCL	0.3 g
$MgCl_2$ $6H_2O$	0.4 g
NH_4Cl	0.3 g
KH_2PO_4	0.2 g
$CaCl_2$ $2H_2O$	0.15 g
Distilled water	970 mL

Autoclave at 121°C for 60 min
Cool and stir under atmosphere of 80% N_2 / 20% CO_2

Solution 2 - Trace Elements = Could use Wolfe's

$FeCl_2$ $4H_2O$	1.5 g
H_3BO_3	0.060 g
HCl (25%)	6.5 mL
$CoCl_2$ $6H_2O$	0.120 g
$MnCl_2$ $4H_2O$	0.100 g
$NaMoO_4$ $2H_2O$	0.025 g
$NiCl_2$ $6H_2O$	0.025 g
$ZnCl_2$	0.070 g
$CuCl_2$ $2H_2O$	0.015 g
Distilled water	993 mL

Autoclave for 20 min at 121°C

Solution 3 -

Na_2SeO_3	0.003 g
NaOH	0.5 g
Distilled water	1000 mL

Autoclave for 20 min at 121°C

Solution 4

$NaHCO_3$	8.5 g
Distilled water	100 mL

Saturate with CO_2 and Filter Sterilize

Solution 5

Na_2S $9H_2O$	12 g
Distilled water	100 mL

Autoclave at 121°C for 20 min in screw-capped bottles with an OFN atmosphere

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MIX THE SOLUTIONS = BASAL MEDIUM

Solution 1	970 mL

Solution 2   1 mL
Solution 3   1 mL
Solution 4   30 mL
Solution 5   3 mL

Adjust pH to 7.2 using HCL or Na_2CO_3

Solution 6
Contains electron donors recommended by Pfennig. Composition varies depending on species studied. Ad components one at a time to 100 mL of water

Sodium acetate   20 g
Propionic acid   7 g
n-Butyric acid   8 g
n-Palmitic acid   5 g
Benzoic acid   5 g
Distilled water   100 mL

Adjust to pH 9 with NaOH
Autoclave in screw-capped bottles at 121°C for 20 min

Add 10 mL of the appropriate electron donor solution to 1 L basal medium

Solution 7 - vitamin solution = Could use other Vitamin Sol
Vitamins may change depending on requirements

Biotin   1 mg
p-Aminobenzoic acid   5 mg
Vitamin B_{12} 5 mg
Thiamine   10 mg
Distilled water   100 mL

Filter sterilize
Add 1 mL to 1 Liter of the basal medium

Solution 8
Distilled water   100 mL
Isobutyric acid   0.5 g
Valeric acid   0.5 g
2-Methylbutyric acid   0.5 g
3-Methylbutyric acid   0.5 g
Succinic acid   0.6 g
Caproic acid   0.2 g

Autoclave at 121°C for 20 min
Add 1mL mL to 1 Liter of the basal medium

Solution 9
Prepare just before use. Deoxygenate the distilled water before adding the sodium dithionite

Distilled water   100 mL

315

Sodium dithionite   3 g

Filter sterilize
Place in scew-capped bottles under an OFN headspace
Add 1mL mL to 1 Liter of the basal medium

8. Sulfate Reducer Media (PM1)

Postgate *et al.* (1963)

This medium can be prepared and autoclaved in screw cap bottles. When made, this medium will contain a precipitate.
Made to 1 Liter

	g/L
KH_2PO_4	0.5
$CaSO_4$	1.0
NH_4Cl	1.0
$MgSO_4\ 7H_2O$	2.0
Sodium lactate	3.5
Yeast extract	1.0
$FeSO_4\ 7H_2O$	0.5
Ascorbate	0.1
Thioglycollate	0.1
NaCL	as needed

Adjust to pH 7.0-7.5

Autoclave at 121 C for 15 min

9. Sulfate Reducer Media (PM2)

Postgate *et al.* (1963)

This medium contains no reducing agents. Medium is autoclaved, then cooled and maintained under anaerobic gas.

Made to 1 Liter

	g/L
KH_2PO_4	0.5
Na_2SO_4	4.5
NH_4Cl	1.0
$MgSO_4\ 7H_2O$	0.06
$CaCl_2\ 6H_2O$	0.06
Sodium lactate	6.0
Trisodium citrate	0.3
Yeast extract	1.0
$FeSO_4\ 7H_2O$	0.004
NaCL	as needed

Adjust to pH 7.5

Autoclave at 121 C for 60 min

10. USGS Media

Recipes for USGS HA Med, IR USGS, H2 Methano and A Methano media were developed at the USGS in Reston, VA, and prepared by Elizabeth Jones. For information on the techniques for USGS media preparation, see:

http://water.usgs.gov/nrp/microbiology/index.html

http://water.usgs.gov/nrp/microbiology/link_feiiimedia.html

11. Growth Media for *T. Ferrooxidans*

Prepare: Solution (A)
Made to 500 mL

	g/L
$(NH_4)SO_4$	3
KCl	0.1
K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.5
$Ca(NO_3)_2$	0.01

Then add:

10N H_2SO_4	1 mL

Prepare: Solution (B)
Add 44 g $FeSO_4 \cdot 7H_2O$ to 500 mL H_2O
Filter sterilize to purify

Prepare: Final Solution
Mix Solution (A) and Solution (B) at a 1:1 ratio.

Appendix III – Additional Solutions

1. TE buffer

Add:
10 mL	10 mM Tris (bring to pH 7.5 with HCl)
2 mL	1 mM EDTA (pH 8.0)

TE is a commonly used buffer for preventing degradation of DNA or RNA. It is composed

of Tris (pH buffer) and EDTA (anti-chelating agent). TE is made per litre with 10 mL 1M

Tris-Cl at pH 7.5 and 2 mL 500mM EDTA at pH 8.0.

2. Phosphate Buffered Saline Solution

Madeto 500 mL as a 10x concentrated stock solution.

Add:

NaCl	4.0 g
KCl	0.1 g
Na_2HPO_4	0.72 g
KH_2PO_4	0.12 g

3. Artificial Seawater(ASW) / Formaldehyde Preparation

Madeto 1 L

Dissolve the following (in the order stated) in formaldehyde diluted to 2% in filter-sterilized deionized water:

NaCl	27.4 g
KCl	0.77 g
$MgCl_2$	11.21 g
$NaHCO_3$	0.12 g
$CaCl_2$	2.4

Reference:
Cragg, B.A., Parkes, R.J., Fry, J.C., Herbert, R.A., Wimpenny, J.W.T., Getliff, J.M. 1990. Bacterial Biomass and Activity Profiles within Deep Sediment Layers. *In*: Suess, E., von Huene, R., *et al*. (Editors). *Proceedings of the Ocean Drilling Program, Scientific Results, Volume 112.*

4. Citric Acid Buffer for Acridine Orange Peroration

(A) Add 10.5 g Citric Acid to 500 mL filter-sterilized deionized water
(B) Add 2 g of NaOH to 500 mL filter-sterilized deionized water

To make the final solution:
100 mL (A) + 59 mL (B)

After combining (A) and (B), filter sterilize the final solution.

Reference:
Cragg, B.A., Parkes, R.J., Fry, J.C., Herbert, R.A., Wimpenny, J.W.T., Getliff, J.M. 1990. Bacterial Biomass and Activity Profiles within Deep Sediment Layers. *In*: Suess, E., von Huene, R., *et al.* (Editors). *Proceedings of the Ocean Drilling Program, Scientific Results, Volume 112.*