To the Graduate Council:

I am submitting herewith a thesis written by Udit Minocha entitled "Bioavailability of Dissolved Organic Carbon in Surface Water by Surface and Subsurface Environmental Bacteria." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

Dr. Gary S. Sayler, Major Professor

We have read his thesis
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of the Graduate School

Bioavailability of Dissolved Organic Carbon in Surface Water by Surface and Subsurface Environmental Bacteria

A Thesis Presented

for the

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Udit Minocha

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Study without thinking is worthless;

thinking without study is dangerous.

- Confucius

Dishonesty is knowing but ignoring the fact

that the data are contradictory.

Stupidity is not recognizing the contradictions.

- Anonymous

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Briess malting, L. D. Carlson, Edme, Craftsman tools and Brooks Athletics helped me to regain my sanity by brewing, fixing mechanically-challenged vehicles, and running. I might add there was no write-up involved after these activities were completed.

ABSTRACT

Metabolism of naturally occurring dissolved organic carbon (DOC) in surface water was examined. Environmental bacteria isolated from the Savannah River Site, (SRS) Aiken, SC, (subsurface soil) and from Georgetown, SC (surface water) were grown in a minimal medium to which surface water containing DOC was added. Three DOC concentrations for each pure bacterial isolate were tested. The negative control, which was the same for all bacteria, consisted of minimal salts in place of the surface water. The two other concentrations of DOC were 2 and 10 mgC-DOC/l. The initial inoculum was ca. 10^2-10^3 CFU/ml. Bacteria were plated every 24 h. The cells in 0 mgC-DOC/l routinely grew to 10^5-10^6 CFU/ml. The 2 and 10 mgC-DOC/l were only metabolized by two other bacteria, B0612 and G13 from the Savannah River Site, Aiken, SC, and Georgetown, SC.

Gram stains and the Biolog assay were used to identify the environmental isolates. Most of the bacteria from SRS were Gram negative (*Pseudomonas* and *Acinetobacter*) whereas surface water isolates from Georgetown were Gram positive (*Bacillus* and *Corynebacterium*). In general, surface water bacteria were able to metabolize more substrates in the Biolog assay than subsurface bacteria. Subsurface bacteria metabolized primarily carboxylic acids and amino acids. Surface water bacteria metabolized primarily carbohydrates. There was no correlation between the number or kind of substrates metabolized in the Biolog assay and their ability to metabolize naturally ocurring DOC.

TABLE OF CONTENTS

I. INTRODUCTION 1

II. LITERATURE REVIEW 3

ROLE AND DISTRIBUTION OF MICROBES IN THE GROUND 3
HUMIC SUBSTANCES 4
FORMATION OF HUMIC SUBSTANCES 5
STUDIES ON DOC METABOLISM 7
NATURAL DEGRADATION OF ORGANIC POLLUTANTS 10

III. MATERIALS AND METHODS 12

BACTERIAL ISOLATES 12
GRAM STAIN, BACTERIAL IDENTIFICATION AND METABOLIC PROFILES 14
SURFACE WATER AND INOCULUM PREPARATION 15
DISSOLVED ORGANIC CARBON ANALYSIS 16
STANDARD CURVE/SPECTROPHOTOMETER DETECTION LIMIT 16
BIOAVAILABILITY ASSAY (DOC) BY OPTICAL DENSITY 16
BIOAVAILABILITY ASSAY (SUCCINATE) BY OPTICAL DENSITY 17
BIOAVAILABILITY ASSAY (DOC) BY COLONY FORMING UNITS (CFU) 17

IV. RESULTS 19

GRAM STAIN, BACTERIAL IDENTIFICATION AND METABOLIC PROFILE 19
STANDARD CURVE/SPECTROPHOTOMETER DETECTION LIMIT 19
BIOAVAILABILITY ASSAY (DOC) BY OPTICAL DENSITY 33
BIOAVAILABILITY ASSAY (SUCCINATE) BY OPTICAL DENSITY 33
BIOAVAILABILITY ASSAY (DOC) BY COLONY FORMING UNITS (CFU) 36

V. DISCUSSION 37

STANDARD CURVE/SPECTROPHOTOMETER DETECTION LIMIT 39
BIOAVAILABILITY ASSAY (DOC AND SUCCINATE) BY OPTICAL DENSITY 40
BIOAVAILABILITY ASSAY (DOC) BY CFU 41

VI. CONCLUSION 48

LITERATURE CITED 49

APPENDICIES 57

APPENDIX A 58
APPENDIX B 59

VITA 61

LIST OF FIGURES

Figure 1. Separation of soil organic matter into the different humic fractions. 6

Figure 2. Flowchart of steps involved in sample processing and experiment setup for the the testing of hypothesis. 13

Figure 3. Standard Curve (a=A0562; b=C0661) Comparing turbidity between the Gilford Spectrophotometer and the 96 well plate reader 31

Figure 4. Bioavailability assay (succinate) by optical density 34

Figure 5. Growth of A0562, subsurface bacteria from Savannah River Site, SC grown in surface water from Georgetown, SC. 42

Figure 6. Growth of A1241, subsurface bacteria from Savannah River Site, SC grown in surface water from Georgetown, SC 43

Figure 7. Growth of B0612, subsurface bacteria from Savannah River Site, SC grown in surface water from Georgetown, SC. 44

Figure 8. Growth of G13, surface water bacteria from Georgetown, SC grown in surface water from Georgetown, SC. 45

LIST OF FIGURES

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Figure 2. Flowchart of steps involved in sample processing and experiment setup for the the testing of hypothesis. 13

Figure 3. Standard Curve (a=A0562; b=C0661) Comparing turbidity between the Gilford Spectrophotometer and the 96 well plate reader 31

Figure 4. Bioavailability assay (succinate) by optical density 34

Figure 5. Growth of A0562, subsurface bacteria from Savannah River Site, SC grown in surface water from Georgetown, SC. 42

Figure 6. Growth of A1241, subsurface bacteria from Savannah River Site, SC grown in surface water from Georgetown, SC 43

Figure 7. Growth of B0612, subsurface bacteria from Savannah River Site, SC grown in surface water from Georgetown, SC. 44

Figure 8. Growth of G13, surface water bacteria from Georgetown, SC grown in surface water from Georgetown, SC. 45

I. INTRODUCTION

There has been increased interest in the microbiology of the subsurface in the last few decades. Examination of the subsurface environment at the microscopic level reveals a diverse microbial community (Albrechtsen, 1992; Balkwill, 1989; Hirsch, 1983; Stetzenbach, 1986). It is known that although surface and ground water contain low carbon concentrations, bacteria still are able to exist at low densities (Sepers, 1977; van der Kooij, 1980). Bacteria have been found in the ground at depths as low as 600 meters (Haldeman, 1993; Thomas, 1992).

Subsurface bacteria must obtain carbon from their environment to meet their nutritional requirements. Biodegradation of natural compounds (i.e., glucose, amino acids, organic acids) in heterotrophic activity assays under aerobic conditions indicated that bacteria had the capability to metabolize different kinds of substrates (Capone, 1994; Ventullo, 1985; Ward, 1985). These biodegradation studies were done in the laboratory with ^{14}C-labeled substrates. Mass balance reactions were setup to account for the substrates by assimilation into biomass and mineralization to $^{14}CO_2$. It is important to note that the substrates used in the growth and biodegradation studies do not necessarily reflect the true nature of the organism(s). The substrates used are easily degraded by the mixture of heterotrophs and are not usually found in high concentrations especially in the subsurface environment. The inability to accurately simulate the natural conditions and communities in the laboratory makes it difficult to extend the findings to a large scale field environment.

The predominant form of carbon in the environment exists in the form of humified organic matter including dissolved and/or particulate organic carbon (DOC or POC; Muller-Wegener, 1988). The different stages of organic matter humification dictate the ease of metabolism. In general, organic matter at an early stage of humification is metabolized easier than at a later stage. In 1978, Sayler reported a correlation between heterotrophic activity and the concentration of available dissolved organic carbon (DOC).

Recent research has resulted in the recovery of many bacterial strains from subsurface and ground water environments. It was of primary intent to understand the physiology and nutritional ecology of these strains in order to evaluate their fundamental roles in biogeochemical processes and for potential application in environmental protection and restoration.

The specific objective of this study was:

to determine which fraction of DOC in surface water was metabolizable by heterotrophic bacteria previously isolated from an uncontaminated subsurface environment.

The data obtained from research on this objective will provide an important foundation and increased knowledge in the potential of organic pollutant remediation by endogenous bacteria *in situ.* The energy source required by bacteria, in some cases, would come not from organic pollutants, rather, it would come from the DOC found in the subsurface environment. A better understanding of cell physiology and the biotic/abiotic interactions may result in lower cleanup costs and improved remediation results.

II. LITERATURE REVIEW

ROLE AND DISTRIBUTION OF MICROBES IN THE GROUND

Initial investigations were conducted from the rhizosphere to about 20m below the surface (Balkwill, 1989; Thomas, 1992). Below those depths, it was felt that contamination from drilling operations was highly likely. It was not until after 1980 that aseptic drilling procedures for the shallow subsurface were developed well enough that studies on mucrobial ecology could be effectively conducted (Belion, 1988). Recently, specialized drilling techniques have been developed for aseptic drilling of the deep subsurface (Leach, 1991). Bacteria are the predominant type of microorganism present in both the shallow and deep subsurface environments (Frederickson, 1989; Fliermans, 1989; Ghiorse, 1988) although protozoa, fungi and algae have been detected in the shallow subsurface (Ghiorse, 1988).

The bacteria found in the subsurface are highly diverse and have a broad array of metabolic capabilities (Lee, 1988; Ventullo, 1985). This diversity resembles the complex and heterogenous nature of the environment. The variability of the environment makes it difficult to predict microbial processes. However, studies have shown that bacteria are able to grow and survive in oligotrophic environments. They are also able to metabolize a variety of natural and xenobiotic compounds (Balkwill, 1989; Swindoll, 1988).

Typically, the first set of experiments done to characterize bacteria from the environment are cell number determinations, the ability to metabolize a wide range of organic compounds, and cell activity (usually with radiolabeled compounds). The number and activity of microbes may be higher in sandy sediments than in clay (Jiménez, 1990; Thomas, 1992). Bacterial counts will vary depending upon the enumeration technique used with viable counts being usually lower than acridine orange direct counts (AODC). It is estimated that only 0.01 to 50% of the bacteria can be recovered by plating on solid media (Lee, 1988). Albrechtsen (1992) reported viable counts and AODC from subsurface sediment samples ranging from 0.5-1,203x10^3 colony forming units (CFU)/g dry weight and 1.7-147x10^7 cells/g dry weight, respectively. Ventullo (1985) reported viable counts and AODC from subsurface sediment samples ranging from 0.31-8.6x10^3 cells/ml and 0.036-0.21x10^6 cells/ml, respectively. Microbial activity as measured by ^{3}H-thymidine and ^{14}C-labelled substrate incorporation can be a measure of bacterial

productivity. Capone (1994) found, in ^{14}C-glucose uptake experiments, that almost 90% of the labelled glucose was incorporated into biomass. Other studies have also reported similar results (varied from 1.4-60.7x10^4 cells/(g dry weight per day).

HUMIC SUBSTANCES

The decay products of plant and animal material is referred to as natural organic matter (NOM). It is the main source of organic carbon for respiration. NOM, which exists as particulate organic carbon (POC) and dissolved organic carbon (DOC), are important sources of nutrition for bacteria. NOM may be classified into two broad categories: nonhumic and humic substances.

All of the NOM in soil, with the exception of source identifiable materials (nonhumic compounds), is called humus (Sposito, 1989). Humus formation is not fully understood. The nonhumic compounds are source identifiable materials and include compounds such as carbohydrates, proteins/peptides/ amino acids, fatty acids, waxes, and other low molecular weight compounds. This group of compounds is relatively labile and easily metabolized by many microorganisms in soils or surface waters. The humic substances are relatively resistant to microbial degradation (Wetzel, 1983). They are characterized by long residence times in soil and surface/ground water.

Humic substances make up about 85% of soil organic matter (Smith, 1993). The two major components of humic substances are humic acid and fulvic acid. Their definitions are operationally defined as they are separated by their acid/base solubility characteristics (Fig. 1). Humic acids are soluble at high pH and are of higher molecular weight than fulvic acids. The latter are soluble at low pH and are generally of lower molecular weight (Metting, 1993).

The properties of humic substances differ from each other. However, a few generalizations can be made. Humic substances are large, acidic molecules (MW=500 to >250,000 Daltons) and are yellow to brown in color (Hedges, 1988). They have complex chemical structures that can not be easily characterized. Only 10-20% of the molecular structures in the polymerized form can be recognized as coming from recognizable biochemicals (Hedges, 1988). Humic substances contain large amounts of nitrogen. In living organisms, nitrogen is most commonly found in proteins and amino acids which do not tend to recombine spontaneously in the environment. Since amino acids are a minor component of humic

Soil Organic Matter Separation

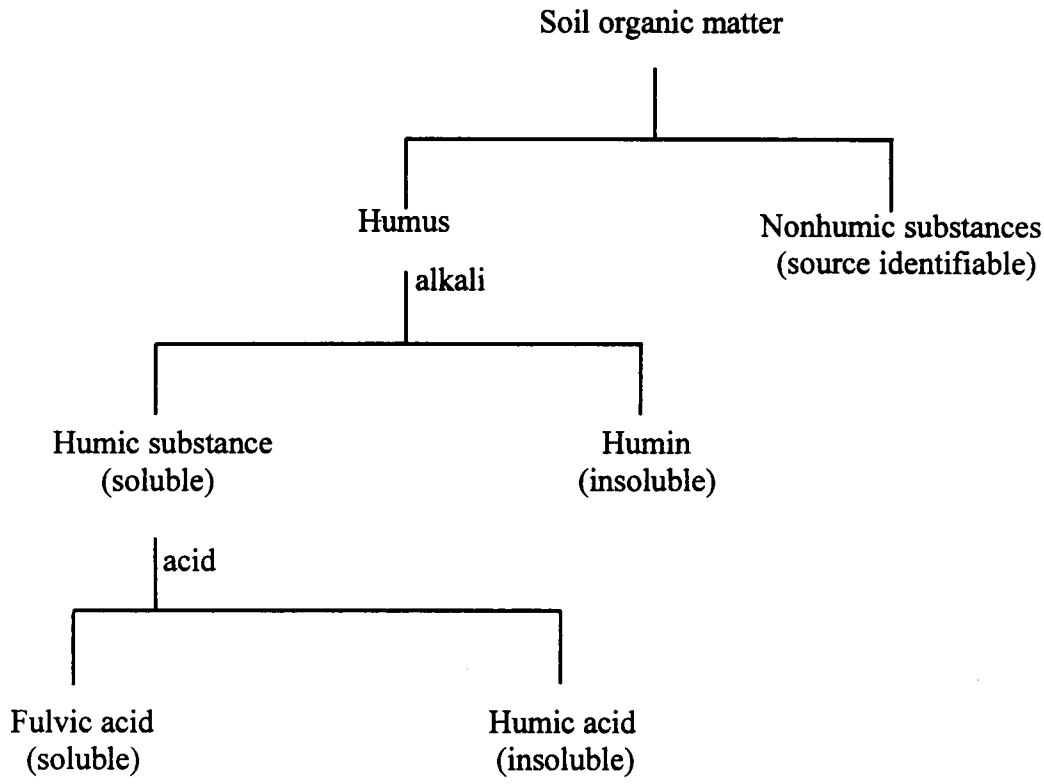


Figure 1. Separation of soil organic matter into the different humic fractions.

polymers, microbial degradation and polymerization are required for the reactions (Thurman, 1985). The ubiquity of humic substances in the environment indicates that although they may not be the optimal carbon and energy source for bacteria, bacteria must be able to metabolize them for their survival.

FORMATION OF HUMIC SUBSTANCES

Humic substances are compounds that are formed by the degradation and polymerization of large organic molecules. Although these mechanisms are unclear, there are models to explain their formation. Humification models are based on the fact that plant and animal material is degraded by microorganisms. It is not known if polymerization occurs by the microorganisms to form biopolymers and is then further modified in the environment or if the degraded remains recombine spontaneously in the environment (Hedges, 1988; Stevenson, 1982). It is important to note that other organisms besides bacteria (plankton, fungi, multicellular organisms) play a role in humification.

There are two schools of thought on the formation of humic substances: the lignin-protein theory by Waksman (1932) and the polyphenol theory by Flaig (1966) and Kononova (1966). The lignin-protein theory states that the nitrogen in humic acids results from the condensation of modified lignin and protein that originated from microorganisms. Waksman compared the similarities between lignin, the building blocks, and humic acid (adapted with permission from Stevenson, 1982).

Both lignin and humic acid:

1) are recalcitrant and are difficult to break down by bacteria and fungi.
2) are slightly soluble in alcohol and pyridine.
3) are soluble in base but are precipitated by acids.
4) contain methoxyl (OCH_3). The methoxyl group in both diminish with stage of decomposition.
5) are acidic, able to combine with bases, and are characterized by their base exchange capacity. Although to a different quantitative extent.
6) When lignins are warmed with aqueous alkali, they are transformed into methoxyl-containing humic acids.
7) Humic acids have properties similar to oxidized lignins.

Although the lignin-protein theory is now considered obsolete, it can not be totally ruled out. In aerobic, well-drained soils, the fungi that degrade lignin, degrade it into low molecular weight products. In poorly drained soils, however, modified lignins predominate (Stevenson, 1982).

The polyphenol theory is the accepted paradigm. There are two pathways in this model. In the first pathway, lignin is still the origin. However, microorganisms enzymatically convert the lignin to quinones which polymerize with or without the presence of amino acids. The products are humiclike macromolecules. In the second pathway, microorganisms synthesize polyphenols from nonlignin sources (cellulose). The polyphenols are enzymatically converted to humiclike substances with or without amino acids.

STUDIES ON DOC METABOLISM

Studies on the influence of organic nutrient concentration on microorganisms were initially performed during the early and mid-ninteen hundreds on surface water: fresh and salt water. Krough (1931) cited the work of Putter (1909) studied the bioavailability of dissolved organic matter (DOM), which includes DOC and nitrogen and phosphorous containing compounds, to lower aquatic invertebrates (*Collozoum inerme*, a sea water protozoan). Putter concluded that DOM is an important nutrient source for these organisms. Krough (1931) criticized these findings stating that there was no evidence that DOM served as a nutritional source for lower invertebrates. The primary reason being the lack of evidence indicating that membranes of filter feeders are permeable to DOC. The major argument was that the main source of organic carbon could be from particulate organic carbon.

Kuznetsov (1970) translated into English (from Russian) the results of Sorokin and Yarovitisina (1964) had shown that bacteria can grow and multiply in carbon-poor environments. There has been no consensus as to the minimal carbon concentration required to support bacterial growth (Hucker and Carpenter, 1927; and Bigger, 1937). ZoBell and Grant (1942, 1943) were able to grow marine bacteria in a mineral media where different carbon sources were used at concentrations ranging from 0.25 to 10.0 mg/L. Their opinion was that 0.1 mg/L should not be considered the lowest concentration of carbon that can support growth. However, they did not attempt concentrations below 0.25 mg/L because any labile molecule of food that contacts a bacterial cell should be utilizable. An organism can not live long in an environment that does

not provide sufficient nutrients. If the energy supply within an organism gets too low then death of the cell is imminent. Based on these reports it can be concluded that bacteria in the environment alternate between feast and famine states but are most likely to be found in the starvation state. Waksman and Carey (1935) showed that a few hundred bacteria can multiply to several millions of cells/ml in sea water stored in the laboratory. Also, they showed that nitrogen is a limiting factor in bacterial cultures. Kuznetsov (1970) translated into English (from Russian) the results of Vinberg and Yarovitsina (1946) which confirmed the results of Waksman (1935) and ZoBell (1936) when they reported an increase in the number of bacteria by direct microscopy in jars of stored water from lakes Beloe and Svyatoe in the former USSR. They also reported a decrease in the oxygen content in these storage jars. These results indicated that respiration was occurring and there was metabolizable organic carbon in the water from those lakes.

Around the mid-1930's, much work was done in deducing the metabolic pathways. It was helpful in knowing that most organisms had similar metabolic processes. This information was a boon to researchers studying the degradation of natural organic matter as a carbon and energy source. Birge and Juday (1934) reported that American lakes contained 3-50 mg organic matter per liter water. They reported that water-soluble organic compounds formed by the autolysis of phytoplankton are composed of proteins/amino acids, uronic acids, pectins, and humic substances. The inability to maintain cultures at a particular growth phase for long periods inhibited researchers in simulating environmental culture conditions.

Research in growth kinetics by Monod indirectly impacted the study of carbon utilization. Monod mathematically expressed growth kinetics under steady state conditions. Equations developed during the 1950's were useful for the development of the chemostat. In a chemostat, it was possible to maintain bacteria at a particular physiological stage. Kinetic theories of nutrient-limited cell population growth and the relation between the specific growth rate and the concentration of the limiting nutrients (Powell, 1968; van Unden, 1969). Usually work was done with cultures in stationary phase because cells are most often in a starved state in the environment. Besides advances in microbiology, analytical chemistry increased the sensitivity and ability to measure humic substances.

Techniques such as the ability to separate humic substances by molecular size, organic carbon composition analysis, and trihalomethane formation potential, made it possible to study and characterize humic substances. Geller (1985) addressed the issue of the processes that lead

to aquatic humification. It was found that the labile low molecular weight substances combine with the higher molecular weight substances and promote humification forming increasingly recalcitrant compounds. The labile low molecular weight substances combine with the higher molecular weight substances promote humification forming increasingly recalcitrant compounds. A compound at a later stage of humification will be more recalcitrant than one in an earlier stage. However, the question of knowing what fractions of DOC are available to bacteria as a source of carbon and energy remains unanswered. This is important to understand the role microbes have in the food web (Meyer, 1987). Carbon in the environment may be metabolized as a carbon and energy source, to selected groups of bacteria. It could be that as DOC is metabolized its hydrophobicity/hydrophilicity changes. It may be that as carbon chains or functional groups are modified or broken off the humic substances, the phobicity of the NOM alternates between hydrophobic and hydrophilic, rendering the compound bioavailable or unavailable. Another group of bacteria in the same environment may be able to utilize a different fraction of NOM, namely the one that became unavailable to the first strain. The ability of an organism to metabolize a compound may be related to the degree of humification.

The techniques used currently to measure uptake and activity or growth are similar to the techniques that were used from the middle part of the century. Heterotrophic distributions and utilization of low concentrations of organic substrates are determined by plate counts, direct counts, radiolabel incorporation, O_2 uptake, and CO_2 liberation, organic carbon uptake. The two simple ways to measure the metabolism or incorporation of DOC from the environment are to compare the difference between initial and final DOC concentration in a system. The second way is to follow cell density over time and to compare the initial and final cell density (Servais, 1987). A variation to these methods are to use radiolabelled carbon and check for the internalized radiolabel. Parsons and Strickland (1962) were the first to use uniformly labelled ^{14}C glucose and acetate.

The assimilable organic carbon (AOC) assay was developed to indicate growth potential rather than measurement of biodegradable carbon since the limiting nutrient may not be carbon (van der Kooij, 1977). The AOC assay is based on measuring the growth of a specific bacterium in the water sample. *Pseudomonas fluorescens* was selected because of its capability of metabolizing a wide variety of organic compounds. Also, this organism was able to grow on a simple nitrogen source, did not require growth-stimulating substances (vitamins, cofactors), grew rapidly, and was easy to maintain as a pure culture. The assay involves the preparation of a

preculture which was the inoculum that was added to the water containing AOC. The preculture was prepared by introducing the bacteria (500 CFU/ml) into autoclaved tap water with 1 mgC-acetate/l from a turbid suspension of an overnight agar slant. This preculture was incubated for a week at 25°C and resulted in maximum colony counts of $4x10^6$ CFU/ml. After the week long incubation, the preculture was stored at 6°C and could be stored for several months with only a slight decrease in CFU counts. The precultures were used to inoculate water samples with an initial cell density of 50-500 CFU/ml. The experiment entails adding the preculture to the test water and monitoring growth by periodic (daily) plate counts. In another report, van der Kooij (1980) studied the growth of *Aeromonas hydrophila* at low substrate concentrations added to tap water in the same way. Colony forming units (CFUs) were used to determine the generation time and biomass. In both experiments (van der Kooij, 1977; 1980) a linear relationship was observed between the carbon concentration and the maximum growth rate. It must be noted that not all bacteria can utilize all forms of carbon. A form of carbon easily utilized by one bacterium may be recalcitrant to another.

NATURAL DEGRADATION OF ORGANIC POLLUTANTS

The Clean Water Act (1970) enacted by the U.S. government publicized the need for stringent controls for the release of industrial pollutants into the environment. The Environmental Protection Agency's (EPA) Council on Environmental Quality reported in 1981 that the ground water of at least one state in each of the 10 EPA regions has organic compound pollution. One of the first studies done on the biological activity in relation to ground water pollution was by Dunlap and McNabb (1973). This study drew much attention to the fact that biological activity does not solely occur at the rhizosphere even though the subsurface environment is oligotrophic. Researchers have since focused on subsurface sediments to better understand the utilizaton of carbon in the environment by heterotrophic bacteria (Balkwill, 1985; Ghiorse, 1988; Stetzenbach; 1986). A number of techniques are used to remediate polluted ground water: physical containment by barriers, *in situ* treatment by the addition of chemicals or microbes, and withdrawal and treatment by physical, chemical or biological processes. The use of microorganisms in the degradation of pollution, referred to as bioremediation, has created a large area of primary research.

Although some bacteria have the capability to degrade pollutants, they do not necessarily derive energy from them. The main source of energy is most likely the degradation of humic substances found naturally in the environment. The belief is that pollutants in the environment can be degraded if their chemical structures resemble natural organic matter. Microbes have been exposed to a diverse range of natural compounds and have evolved in response to degrade them (Lee, 1988; Aelion, 1989). However, many pollutants have structural features unrecognizable by their enzymatic systems and are unable to be degraded. Bioremediation requires microbes with catabolic enzymes that recognize pollutants.

Characterization of microbes in the subsurface environment is the first step in understanding the degradation of NOM. These findings can then be extended to the degradation of pollutants. Cerniglia (1991) discussed the use of microorganisms in the remediation of hazardous wastes. There are areas where research needs to be done to understand the ecological, chemical and toxicological characteristics of a site, conditions that will ensure the survival of pollutant degrading microbes, development of cell systems (immobilized, encapsulated) to facilitate degradation, coupling of aerobic and anaerobic systems, and optimization of pollutant degradation.

It is important to study the degradation processes of naturally occurring organic matter to build a firm foundation that will aid us in understanding pollutant degradation. Also, the subsurface is a unique environment for the study of interactions of bacteria with their environment. This study has examined subsurface bacteria and their ability to metabolize dissolved organic carbon found in unpolluted surface water.

III. MATERIALS AND METHODS

All bacteria were incubated at 25°C, in the dark and not shaken. Turbidity measurements were made at 590 nm. Glassware was combusted at 550°C to remove carbon adhered to the glass surface (Kaplan, 1993).

BACTERIAL ISOLATES

Several strains of subsurface bacteria were obtained from the Subsurface Microbial Culture Collection (SMCC) at Florida State University, Florida. They are soil bacteria isolated from the Middendorf formation (depth=235-257 m) at Savannah River site (SRS), Aiken, South Carolina. They are designated with a prefix A, B, or D followed by the isolate number. The prefixes correspond to drill sites P28, P24, and C10, respectively (Palumbo, 1990; Hazen, 1990).

Surface water from Georgetown, South Carolina was used to obtain environmental isolates and were designated as Gx (see Figure 2 for flowchart). The Georgetown study site is located on the grounds of the Baruch Research Institute. Surface water was collected in sterile 50ml sample tubes. The sample tubes were stored at 4°C. Although the number of dilutions that were done varied, it was necessary to do 10^{-3}-10^{-5} dilutions to obtain 30-300 colonies per plate. Colonies were differentiated and selected by their pigmentation, appearance, size, and texture. Most of the colonies were white or cream colored and mucoid circular colonies. Most were of the same size after 48 h incubation at 28°C. Bacterial colonies from the spread plates were streaked on nutrient agar plates to obtain pure cultures. Ten-fold serial dilutions were plated on nutrient agar and incubated at 25°C. Colonies were picked and streaked on nutrient agar plates until pure isolates were obtained (Difco, Detroit, MI). Gram stains, Biolog assay, and the bioavailability assay by colony forming units (CFU) were performed. Isolates were streaked for single colonies on nutrient agar (Difco Laboratories, Detroit, MI) plates monthly and held at room temperature.

Experimental Approach

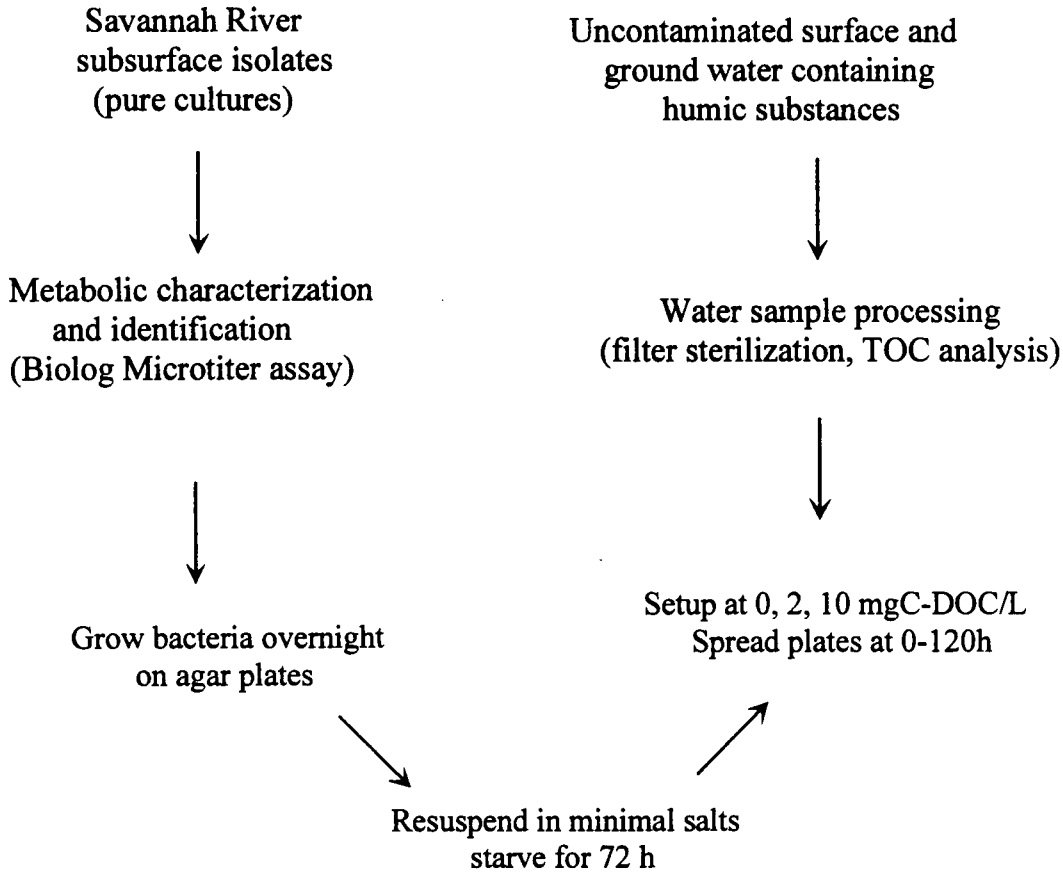


Figure 2. Flowchart of steps involved in sample processing and experiment setup for the testing of hypothesis.

GRAM STAIN, BACTERIAL IDENTIFICATION AND METABOLIC PROFILES

Bacteria were grown on nutrient agar plates less than 24 hours prior to Gram staining (Bartholomew, 1962). A colony from the plate was transferred to a droplet of water on a glass slide and was allowed to air dry. Then the slide was passed through a burner flame to heat fix the bacteria and was allowed to cool. The slide was covered with crystal violet for one minute, rinsed with deionized water, covered with iodine for one minute, and rinsed again. Decolorizer, 67% ethanol (190 proof) and 33% acetone, was added until it ran off the slide colorlessly and the slide was rinsed again a third time. Safranin was added for one minute followed by another deionized water rinse. The slide was blotted dry with bibulous paper and viewed under oil immersion at 1000X magnification (Nikon Model Optiphot).

The Biolog system (Cambridge Technology, Inc., Watertown, MA 02171) was used to determine the metabolic profile and identification of the bacteria (Marello, 1989). Bacteria were grown on nutrient agar plates for 24 hours and were resuspended to an optical density (OD) of 0.2
(ca. 10^8 CFU/ml) in 1X minimal salts media (MSM; Little, 1988; Appendix A). The bacterial suspension was inoculated (150µl) into the wells of the appropriate Biolog (Gram positive or negative plate) 96 well plate. The absorbance was measured at 4 and 24 hours. The Biolog assay was performed on 16 isolates in the appropriate Biolog plate (Gram negative plate, GN, or Gram positive plate, GP).

The Biolog assay is a metabolic test performed in 96-well plates containing 95 known carbon sources, necessary nutrients (proprietary information), and tetrazolium, a colorless oxidation-reduction dye. If the bacteria in a well can oxidize the carbon source, the electrons due to the increased respiration are shuttled via NAD(H) to the electron transport chain and to the dye. The dye is reduced to a purple formazan (resulting in a color change). If the carbon in a well can not be oxidized, there is no formazan produced and the well remains colorless. The wells are scored as a positive (purple) or negative (colorless). the bacterial identification and metabolic profile. In this assay, if the color pattern of the plate tested did not match a pattern in the computer database, the result was that of No Identification (Marello, 1989).

Besides bacterial identification to the genus and species level, similarity and distance were also given. Similarity is used to determine the reliability of the identification. The value of similarity ranges from zero to one. The higher the value the better the identifiation. Similarity is not the same as probability for two reasons. First, the value has been determined from empirical

results and correlated the correctness of the identifications produced with these results. Secondly, summing the probabilities to one ignores the probability that the organism in question is not in the database. In this system, however, it is possible to obtain a number low enough so that the outcome may be one of no identification. Distance is the measure of how far the pattern of the Microplate being tested is from the cluster of the same species to be a perfect match.

SURFACE WATER AND INOCULUM PREPARATION

Surface water was obtained from Georgetown SC, in sterile Nalgene (Nalgene, Rochester, NY) carboys and stored at 4°C. The Georgetown study site is an unconfined sandy coastal-plain aquifer at the Baruch Forest Science Institute (McCarthy, 1993). Surface water was filter-sterilized through a 47mm x 0.2μm polycarbonate filters (Nucleopore, Pleasanton, CA) prior to DOC analysis and addition to complete media.

The bioavailability assay (DOC and succinate) by optical density was setup in the following manner. Bacteria were grown on nutrient agar plates (24-48 h). Care was taken so that the agar surface was not scratched and no agar was present in the MSM containing the resuspended bacteria. The resuspended bacteria (OD 1.0) were transferred to a 100ml ashed bottle containing minimal salts media (MSM; Appendix A; Little, 1988). The bacterial suspensions were incubated overnight at 25°C (not shaking). The overnight incubation was to allow for the metabolism of stored nutrients within the bacteria. After the overnight starvation, the OD was adjusted to 0.6 with 1X MSM. The growth medium consisted of 5 ml bacteria at OD 0.1, 0.6 ml 5% phosphate, 0.3 ml 0.027% $FeCl_3$, MSM (1X final concentration), and surface water (final concentration 2 or 10 mgC-DOC/l).

The bioavailability assay (DOC) by CFU was setup in the following manner. A 72 h starved culture of A0562 was set up as described above (Bioavailability Assay (DOC) by Optical Density) with the following exceptions: bacteria were resuspended in complete media (Appendix A), Balch's vitamin solution (Appendix A; Cote, 1994) at OD 0.5, and the OD after starvation was adjusted to 0.1 (ca. 10^6-10^7 CFU/ml). The suspension was diluted further to reach a final density of 10^2-10^3 CFU/ml in the growth media. The final growth media consisted of 11.0 ml complete medium, 0.8 ml 5% phosphate, 8.0 ml surface water (from stock solutions of 5 and 25 mgC-DOC/l), and 0.2 ml bacteria from a 1000-fold diluted suspension.

Experiments were setup at carbon concentrations of 0, 2, and 10 mgC-DOC/l and 0, 2, 10, 50, 100 and 1000 mgC-succinate/l. The 0 mgC/l sample contained 1X MSM in place of surface water. Stock solutions of surface water or succinate were prepared. Equal volumes of the stock solutions when added to the growth medium yielded the final concentration.

DISSOLVED ORGANIC CARBON ANALYSIS

DOC in surface water was measured by a Model 5000, high temperature combustion total organic carbon analyzer (TOC; Shimadzu). Three standards of phthallic acid were prepared and run before and after each set of water samples. The water samples were acidified to pH 2 and sparged with nitrogen to remove volatile organic carbon prior to analysis. The concentration of all organic carbon substrates was reported as mg-carbon per liter.

STANDARD CURVE/SPECTROPHOTOMETER DETECTION LIMIT

The lower limit of cell density detection was determined by making standard curves of A0562 and C0661, from OD (turbidity) measurements from the spectrophotometer (590nm; path length 1cm; Gilford Response UV-Visible spectrophotometer, Oberlin, OH) and a 96 well plate reader (Cambridge Instruments, Cambridge, MA). Bacteria were grown on an agar plate and transferred to MSM. The cell density was adjusted to OD 1.0. Dilutions were made in stepwise increments: 1:2, 1:5, 1:10, 1:25, 1:50, 1:75, and 1:100. Aliquots (150µl) were transferred to a 96 well microtiter plate and read in the ninety six well plate reader. From each set of dilutions in the plate, 100µl from three wells were diluted separately (ten-fold serial dilutions) and spread plated on nutrient agar plates.

BIOAVAILABILITY ASSAY (DOC) BY OPTICAL DENSITY

The bioavailability of DOC in surface water was assayed by the incorporation of DOC into biomass resulting in the change of OD. An aliquot (150µl) of the complete medium/bacteria solution was transferred to a row (n=8) of wells in a 96-well plate and read on an automated platereader. Initial absorbance readings (590nm; time 0 hour) of each well were taken followed by readings at 24, 48, and 72 hours. The target OD for the start of the experiment was about

0.03. The blanks were the same as the growth media but did not contain bacteria. Instead, additional MSM was added to replace the volume taken up by the bacteria.

BIOAVAILABILITY ASSAY (SUCCINATE) BY OPTICAL DENSITY

A 72 h starved culture of A0562 was set up as described above (Surface Water and Inoculum Preparation) in a 96 well plate containing 150 µl per well (see Bioavailability Assay (DOC) by Optical Density). The six succinate concentrations were 0, 2, 10, 50, 100, and 1000 mgC-succinate/l. The OD was measured every 24 h in a 96 well plate reader. In this assay, the plates containing different concentrations of succinate (2, 10, 50, 100, and 1000 mgC-succinate/l) were treated (solutions were added) with MSM, succinate, $FeCl_3$ and/or phosphate buffer. Nine treatments were done. The 0 mgC-succinate/l served as the control (Table 1).

BIOAVAILABILITY ASSAY (DOC) BY COLONY FORMING UNITS (CFU)

The growth medium containing bacteria (20 ml final volume) was added into 50 ml Erlenmyer flasks. Three concentrations of DOC (final concentrations) were setup for each bacteria: 0, 2, and 10 mgC-DOC/l. Flasks were incubated at 25°C (no shaking) in the dark. Samples were stirred and plated at 24 h intervals starting at 0 h.

Table 1. Experimental design of bioavailability assay (succinate) by optical density. A0562 growing in 96 well plates containing minimal media with succinate as a carbon source giving times of carbon and inorganic nutrient additions.

succinate concentration (mgC-succinate)/l	Times of additions (h)	Sequential carbon and inorganic nutrient additions
0	None	None
2	24 96 120	MSM (2X) 2(mgC-DOC)/l 50(mgC-DOC)/l
10	120	10(mgC-DOC)/l
50	96 120	MSM (2X) 50(mgC-DOC)/l
100	96 144	MSM (2X) MSM (2X); (20ml/l) $FeCl_3$; 40ml/l PO_4
1000	144	MSM (2X); (20ml/l) $FeCl_3$; 40ml/l PO_4

IV. RESULTS

GRAM STAIN, BACTERIAL IDENTIFICATION AND METABOLIC PROFILE

The Gram stain and Biolog results gave tenative identifications on the environmental isolates. There were 7 Gram negative (G-) and 2 Gram postive (G+) bacteria from SRS and 2 G- and 5 G+ from Georgetown.). The Biolog assay output included identification to the level of genus and species and results of similarity and distance. Identifications shown were from the 24h reading. The G- bacteria were most closely matched as: *Acinetobacter*, *Comamonas*, *Haemophilus*, *Klebsiella*, or *Pseudomonas*. The G+ bacteria were most closely matched as *Corynebacterium*, *Bacillus*, or *Arcanobacterium* (Tables 2a and 2b). The subsurface bacterium, from SRS, D0544 (G- rod) had the most accurate match with a similarity of 0.832 and a distance of 0.257 (at 24 h; Table 3) the poorest match was that of G13 (G+ rod) with a similarity of 0.292 and a distance of 11.140 (at 24 h).

The Biolog results indicate a great range in the number of substrates oxidized by the various strains. The subsurface bacteria, from SRS, B0612 metabolized the greatest number of compounds (n=94). The bacterium B0719 from the same site metabolized the least number of compounds (n=2). Tween 40 and tween 80 were the commonly metabolized compounds by subsurface (SRS) and surface water (Georgetown) bacteria. The SRS bacteria primarily metabolized carbohydrates and carboxylic acids, whereas the bacteria from Georgetown preferred carbohydrates (Tables 4 and 5).

STANDARD CURVE/SPECTROPHOTOMETER DETECTION LIMIT

The OD of A0562 and CO661 were measured in the 96 well plate reader and Gilford spectrophotometer (Figs. 3a and 3b). The 96 well plate reader did not detect changes in cell number until an OD of 0.02 ($4x10^7$ CFU/ml; A0562; Fig. 3a) and OD 0.02 ($2x10^6$ CFU/ml; C0661; Fig. 3b).

Comparison of OD measurements between the 96 well plate reader and cell numbers for A0562 (Fig. 3a) show there was little change in OD between $8x10^6$ (OD 0.001) and $1x10^7$ CFU/ml (OD 0.02). At $3x10^7$ CFU/ml (OD 0.03) the curve was linear with respect to OD and CFU's. Similarly, C0661 (Fig. 3b) measured by the 96 well plate reader was not linear until OD 0.006 ($8x10^6$ CFU/ml).

Table 2. Biolog identification of (a) subsurface and (b) surface water bacteria.

Strain	Biolog number @ 4h* @ 24h†	*Genus species* *@ 4h* *@24h*
(a) Subsurface bacteria		
A0562 G- rod	0200-0000-0000-7402-0420-4030-0000-0020 0304-0701-0003-7406-1773-5734-5355-4160	*Pseudomonas aeruginosa* *Acinetobacter genospecies13*
A0631 G- rod	0000-0100-0000-7000-0020-0000-0000-0000 0300-2101-0003-7556-1735-6775-7755-3370	*Pseudomonas corrugata* *Pseudomonas putida B*
A0635 G- rod	0000-0002-0000-0000-0000-0000-0000-0000 0000-2007-0032-0002-0000-0000-0000-0000	*No ID* *Haemophilus somnus*
A1241 G- rod	0000-0000-0000-0000-0000-0000-0000-0000 0302-2102-0003-2007-1323-6775-6174-4010	*No ID* *Comamonas terrigena*
A1270 G- rod	0000-0000-0000-0000-0000-0000-0000-0000 1704-0505-0003-7557-1777-7774-7777-7370	*No ID* *Pseudomonas putida B*
B0612 G- rod	0000-2102-0000-4000-0000-4000-0000-0000 3677-7777-7777-7777-7775-7777-7777-7737	*Pseudomonas syringae* *Klebsiella pneumoniae*
B0719 G+ rod	0000-0000-0000-0000-0000-0000-0000-0000 0020-0000-0000-4000-0000-0000-0000-0000	*Enterococcus casseliflavus* *Corynebacterium jeikeium*
D0455 G+ rod	0020-0000-0000-0000-0000-0000-0000-0000 0030-0000-0000-0000-0000-0200-0000-0000	*No ID* *Corynebacterium jeikeium*
D0544 G- rod	0000-0000-0000-0000-0000-0000-0000-0000 0200-0000-0002-7002-1631-6374-5111-0000	*No ID* *Acinetobacter calcoaceticus*

* The first row of numbers in this column is the Biolog number after 4h reading.
† The second row of numbers in this column is the Biolog number after the 24h reading.

Note: Subsurface bacteria were originally isolated from Savannah River Site, Aiken, SC. Isolates are stored at Subsurface Microbiology Culture Collection (SMCC, Florida State University).

Surface water bacteria were from Georgetown, SC.

Table 2 (continued).

Strain	Biolog number @ 4h* @ 24h†	*Genus species* *@ 4h* *@24h*
(b) Surface water bacteria		
G1 G+ rod	3625-5612-3742-5421-3200-0100-0000-1600 3625-5632-3742-5425-7200-0101-0000-0600	*No ID* *Bacillus coagulans*
G2 G+ rod	1621-1612-3442-7421-3200-0000-0000-0000 1625-5613-1742-5425-3201-0101-0000-0000	*Bacillus subtilis* *Bacillus subtilis*
G3 G+ rod	1627-5636-1743-5425-3200-0351-1210-7600 1625-5616-1743-5425-3201-0141-0000-7600	*Bacillus pumilus* *Bacillus subtilis*
G5 G- rod	2325-1100-4002-3042-4311-5060-2001-6010 0326-2503-0003-7262-0575-4375-3153-7150	*Pseudomonas putida A* *Pseudomonas corrugata*
G13 G+ rod	0020-0000-0001-0000-0001-0000-0000-0000 0034-0206-0000-0400-2000-3353-7210-0016	*No ID* *No ID*
G16 G+ rod	0020-0000-0001-2000-0200-0200-0000-0000 0030-0000-0000-0000-0200-0020-0000-0000	*No ID* *Corynebacterium jeikeium*
G19 G- rod	1424-0612-7553-7003-3200-0000-5000-7000 3566-5672-7575-7223-3200-0000-0000-0000	*No ID* *No ID*

*The first row of numbers in this column is the Biolog number after 4h reading.

† The second row of numbers in this column is the Biolog number after the 24h reading.

Note: Subsurface bacteria were originally isolated from Savannah River Site, Aiken, SC. Isolates are stored at Subsurface Microbiology Culture Collection (SMCC, Florida State University).

Surface water bacteria were from Georgetown, SC.

Table 3. Closeness of Identification.

Strain	similarity @ 4h @ 24h	distance @ 4h @ 24h	substrates metabolized (n=95) @ 4h @ 24h
A0562 G- rod	0.722 0.434	3.328 2.265	4 38
A0631 G- rod	0.453 0.754	1.485 3.713	51 47
A0635 G- rod	Insuf Growth 0.193	Insuf Growth 5.254	22 6
A1241 G- rod	Insuf Growth 0.331	Insuf Growth 8.373	0 32
A1270 G- rod	Insuf Growth 0.587	Insuf Growth 6.447	1 57
B0612 G- rod	0.901 0.753	0.768 3.375	5 94
B0719 G+ rod	0.106 0.235	0.00 4.560	0 2
D0455 G+ rod	Insuf Growth 0.347	Insuf Growth 4.306	1 3
D0544 G- rod	Insuf Growth 0.832	Insuf Growth 2.517	0 22
G1 G+ rod	0.578 0.316	6.592 7.518	32 36
G2 G+ rod	0.492 0.526	6.385 6.755	23 3043
G3 G+ rod	0.476 0.615	8.442 5.951	43 34
G5 G- rod	0.204 0.520	10.165 7.144	22 42
G13 G+ rod	0.156 0.292	2.136 11.140	1 21
G16 G+ rod	0.279 0.768	3.547 3.414	2 4
G19 G- rod	0.174 0.309	14.548 7.625	23 31

Table 4. Substrates metabolized by subsurface isolates from the Savannah River Site, SC in Biolog Gram positive or Gram negative (GP or GN) plates. Shown as percentages and total number of substrates metabolized.

Strain	Compounds metabolized in Biolog GN or GP 96 well plates	carbon source	% and number
A0562 G- rod	acetic acid, *cis*-aconitic acid, citric acid, formic acid, α & γ-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, D, L-lactic acid, malonic acid, propionic acid, quinic acid, sebacic acid, succinic acid	carboxylic acids	62% (15/24)
	D & L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-leucine, L-phenylalanine, L-proline, L-pyroglutamic acid, L-serine, L-theronine, γ-aminobutyric acid	amino acids	65% (13/20)
	tween 40, tween 80	polymers	40% (2/5)
	glucuronamide, alaninamide	amides	66% (2/3)
	urocanic acid	aromatics	5% (1/4)
	bromosuccinic acid	brominated chemicals	100% (1/1)
	putrescine, 2-aminoethanol, 2,3-butanediol	amines	100% (3/3)

Table 4 (continued).

Strain	Compounds metabolized in Biolog GN or GP 96 well plates	carbon source	% and number
A0631 G- rod	D-fructose, α-D-glucose, D-mannose	carbohydrates	11% (3/28)
	acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, α & β-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, D, L-lactic acid, propionic acid, quinic acid, D-saccharic acid, succinic acid,	carboxylic acids	71% (17/24)
	D & L-alanine, L-alanyl-glycine, L- asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, L-serine, L-theronine, γ-aminobutyric acid	amino acids	90% (18/20)
	methyl pyruvate, mono-methyl succinate	esters	100% (2/2)
	tween 40, tween 80	polymers	40% (2/5)
	glycerol	alcohols	50% (1/2)
	succinamic acid, alaninamide	amides	66% (2/3)
	inosine, uridine	aromatics	50% (2/4)
	bromosuccinic acid	brominated chemicals	100% (1/1)
	phenylethylamine, putrescine, 2-aminoethanol	amines	100% (3/3)

Table 4 (continued).

Strain	Compounds metabolized in Biolog GN or GP 96 well plates	carbon source	% and number
A0635 G- rod	maltose, D-mannitol, D-mannose, D-fructose, turanose	carbohydrates	18% (5/28)
	methyl pyruvate	esters	7.9% (1/24)
A1241 G- rod	D-arabitol, D-fructose	carbohydrates	7% (2/28)
	cis-aconitic acid, α & β & γ-hydroxybutyric acid, α-ketobutyric acid, α-ketovaleric acid, D, L-lactic acid, sebacic acid, succinic acid,	carboxylic acids	37.5% (9/24)
	D & L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-proline, L-pyroglutamic acid, L & D-serine, L-theronine	amino acids	70.0% (14/20)
	tween 40, tween 80	polymers	40% (2/5)
	glycerol	alcohols	50% (1/2)
	succinamic acid, alaninamide	amides	66% (2/3)
	urocanic acid	aromatics	25% (1/4)
	bromosuccinic acid	brominated chemicals	100% (1/1)

Table 4 (continued).

Strain	Compounds metabolized in Biolog GN or GP 96 well plates	carbon source	% and number
A1270 G- rod	L-arabinose, D-galactose, α-D-glucose, maltose	carbohydrates	14% (4/28)
	acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, α & β-hydroxybutyric acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid,D, L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid,	carboxylic acids	83% (20/24)
	D & L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline,L-pyroglutamic acid, D & L-serine, L theronine, D,L-carnitine	amino acids	80% (16/20)
	dextrin, glycogen, tween 40, tween 80	polymers	80% (4/5)
	2,3-butanediol, glycerol	alcohols	100% (2/2)
	succinimic acid, glucuronamide, alaninamide	amides	100% (3/3)
	urocanic acid, inosine, uridine	aromatics	75% (3/4)
	bromosuccinic acid	brominated chemicals	100% (1/1)
	phenylethylamine, putrescine, 2-aminoethanol	amines	100% (3/3)
B0612 G- rod	all compounds sustained growth except sebacic acid	all compounds	100% (94/95)
B0719 G+ rod	β-methyl-D-glucoside	carbohydrates	2% (1/44)
	mannan	polymer	1% (1/8)
D0455 G+ rod	L-malic acid	carboxylic acid	8% (1/13)
	tween 40, tween 80	polymers	25% (2/8)

Table 4 (continued).

Strain	Compounds metabolized in Biolog GN or GP 96 well plates	carbon source	% and number
D0544 G+ rod	3–methylglucose, β-methyl-D-glucoside, α-methyl-D-mannoside, palatinose, stachyose	carbohydrates	11% (5/44)
	β & γ-hydroxybutric acid, α-ketovaleric acid, L-malic acid, methyl pyruvate, methyl succinate, propionic acid, pyruvic acid	carboxylic acids	(8/13)
	L-glutamic acid	amino acids	11% (1/9)
	D-lactic acid methyl ester, methyl pyruvate, methyl succinate	ester	100% (3/3)
	glycogen	polymers	12% (1/8)
	glycerol	alcohol	50% (1/2)
	lactamide, alaninamide, succinamic acid	amides	100% (3/3)

Table 5. Substrates metabolized by surface water isolates from Georgetown, SC in Biolog Gram positive or Gram negative (GP or GN) plates. Shown as percentages and total number of substrates metabolized.

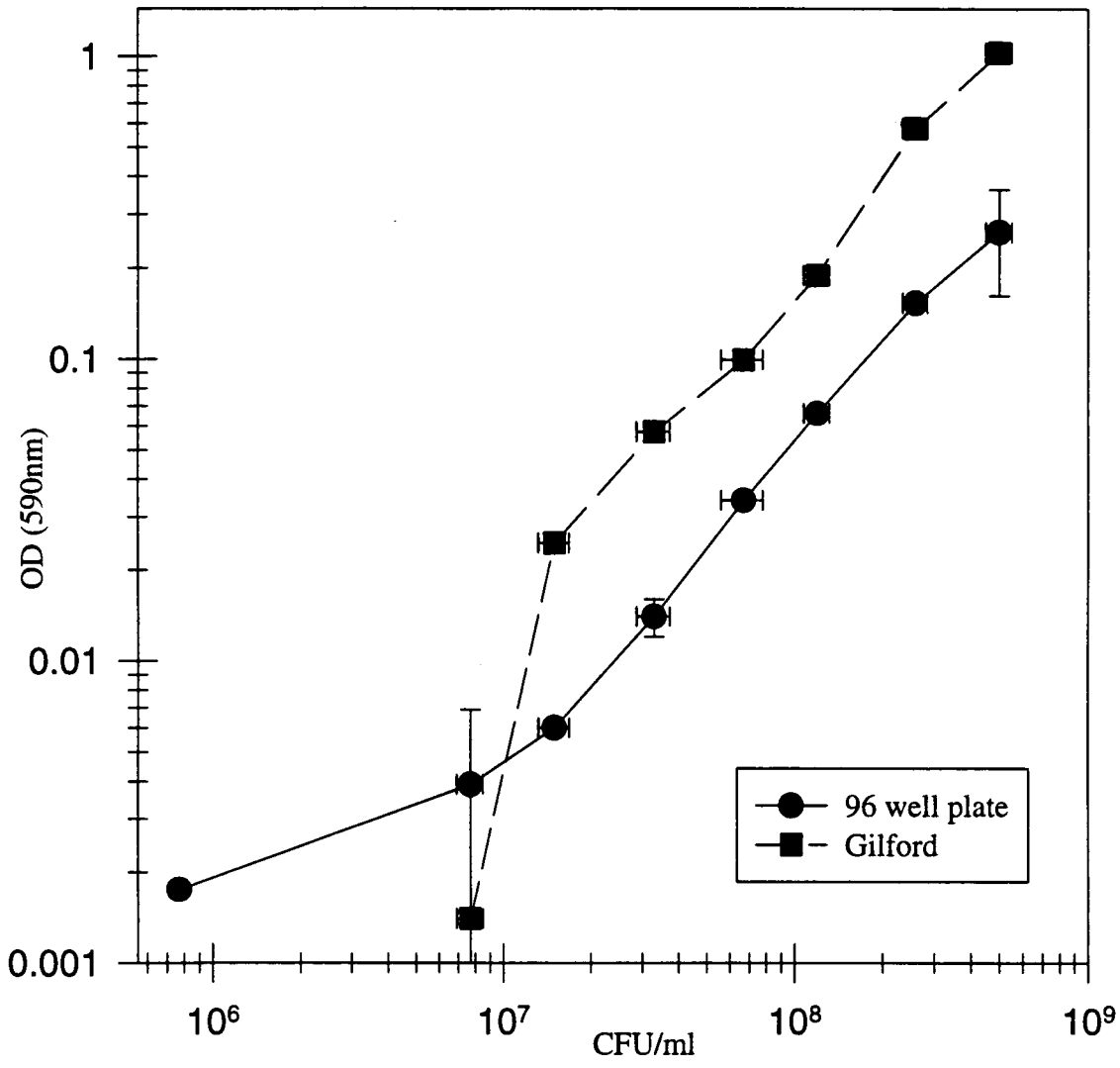
Strain	Substrates metabolized in Biolog GN or GP 96 well plates	carbon source	% and number
G1 G+ rod	N-acetylglucosamine, amygdalin, L-arabinose, arbutin, cellobiose, D-fructose, D-galacturonic acid, gentiobiose, α-D-glucose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, 3-methylglucose, β-methyl-D-glucoside, palatinose, D-psicose, salicin, D-sorbitol, sucrose, D-tagatose, D-trehalose, turanose, D-xylose	carbohydrates	59% (26/44)
	N-acetyl-L-glutamic acid	amino acids	11% (1/9)
	methyl pyruvate	esters	33% (1/3)
	α-cyclodextrin, β-cyclodextrin, dextrin, glycogen	polymers	50% (4/8)
	thymidine, uridine	aromatics	40% (2/5)
G2 G+ rod	N-acetylglucosamine, amygdalin, arbutin, cellobiose, D-fructose, gentiobiose, α-D-glucose, m-inositol, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, 3-methylglucose, β-methyl-D-glucoside, palatinose, D-psicose, salicin, D-sorbitol, sucrose, D-trehalose, turanose, D-xylose	carbohydrates	54% (24/44)
	α-ketovaleric acid	carboxylic acids	8% (1/13)
	N-aceyl-L-glutamic acid	amino acids	11% (1/9)
	methyl pyruvate	esters	33% (1/3)
	β-cyclodextrin, dextrin, glycogen, tween 40	polymers	50% (4/8)

Table 5 (continued).

Strain	Compounds metabolized in Biolog GN or GP 96 well plates	carbon source	% and number
G3 G+ rod	N-acetylglucosamine, amygdalin, arbutin, cellobiose, D-fructose, gentiobiose, D-gluconic acid, α-D-glucose, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, 3-methylglucose, α & β-methyl-D-glucoside, palatinose, D-psicose, salicin, D-sorbitol, sucrose, D-trehalose, turanose, D-xylose	carbohydrates	54% (24/44)
	α-ketovaleric acid	carboxylic acids	8% (1/13)
	β-cyclodextrin, dextrin, glycogen, tween 40	polymers	50% (4/8)
	]adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine	aromatics	100% (5/5)
G5 G- rod	N-acetylglucosamine, N-acetylmannosamine, D-arabitol, cellobiose, L-fucose, α-D-glucose, m-inositol, 3-methylglucose, β-methyl-D-glucoside, α-methyl-D-mannoside, palatinose, D-raffinose, D-ribose, salicin, stachyose, xylitol	carbohydrates	38% (17/44)
	acetic acid, α & β & γ-hydroxybutyric acid, p-hydroxyphenylacetic acid	carboxylic acids	38% (5/13)
	D & L-alanine, L-glutamic acid, glycyl-L-glutamic acid, L-serine	amino acids	55% (5/9)
	methyl pyruvate, methyl succinate	esters	66% (2/3)
	glycogen, inulin, tween 40	polymers	37% (3/8)
	2,3-butanediol, glycerol	alcohols	100% (2/2)
	lactamide	amides	66% (2/3)
	adenosine-5'-monophosphate, fructose-6-phosphate	phosphorylated chemicals	29% (2/7)
	adenosine, 2'-deoxyadenosine	aromatics	40% (2/5)

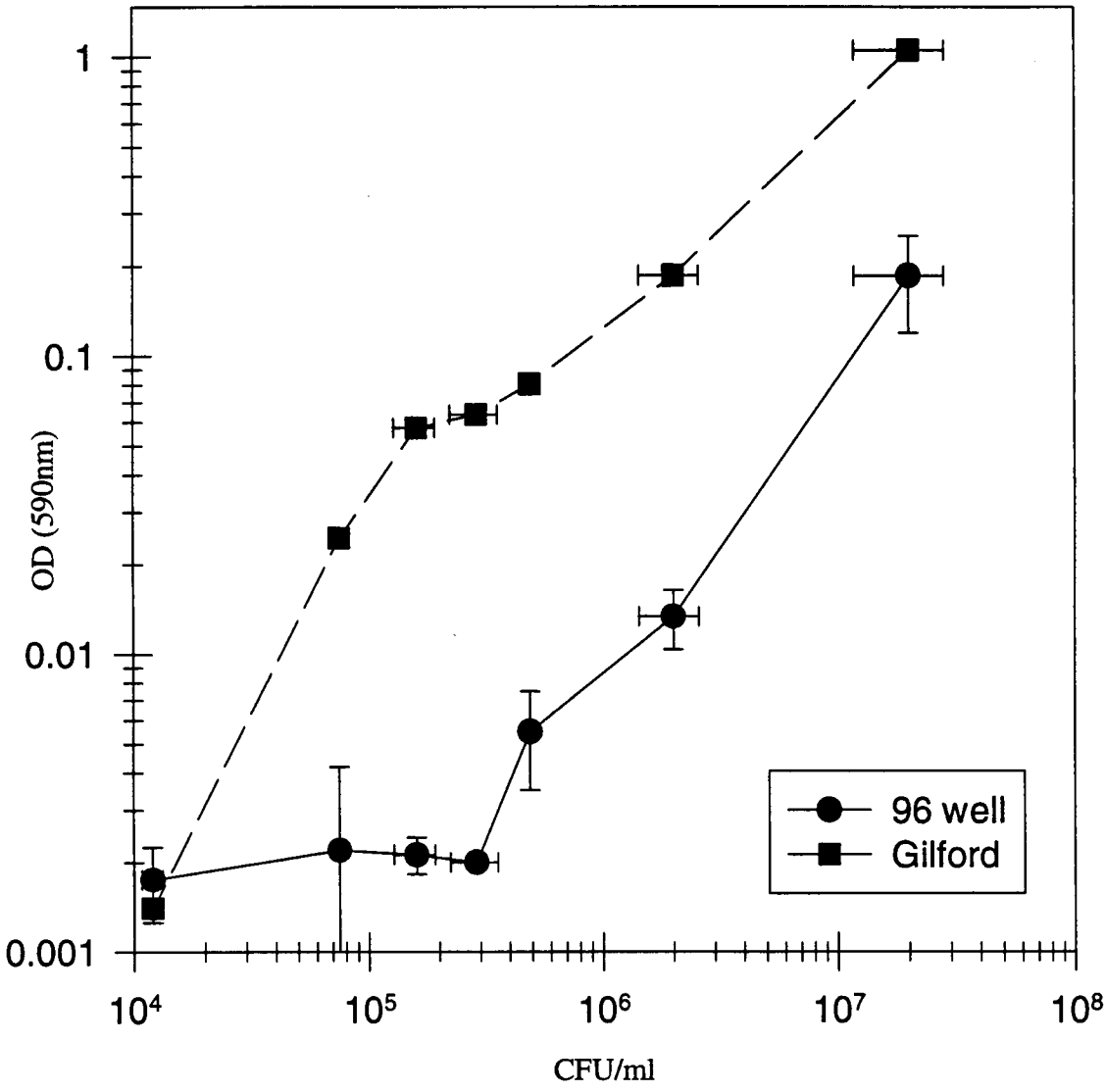
Table 5 (continued).

Strain	Compounds metabolized in Biolog GN or GP 96 well plates	carbon source	% and number
G13 G+ rod	N-acetylglucosamine, D-fructose, D-gluconic acid, α-D-glucose, D-trehalose, D-lactic acid methyl ester, L-lactic acid, L-malic acid, methyl succinate, pyruvic acid, succinic acid	carbohydrates	25% (11/44)
	pyruvic acid, succinic acid	carboxylic acids	15% (2/13)
	L-lactic acid, L-malic acid, D & L-alanine, L-asparagine, L-serine	amino acids	66% (6/9)
	D-lactic acid methyl ester, methyl succinate	esters	66% (2/3)
	tween 40 and tween 80	polymers	25% (2/8)
	alaninamide	amides	33% (1/3)
	fructose-6-phosphate, glucose-1-phosphate, glucose-6-phosphate	phosphorylated chemicals	43% (3/7)
G16 G+ rod	D-xylose	carbohydrates	2% (1/44)
	propionic acid	carboxylic acids	4% (1/24)
	tween 40, tween 80	polymers	40% (2/5)
G19 G+ rod	inulin, mannan, arbutin, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, maltose, maltotriose, D-mannose, D-melezitose, D-melibiose,α & β-methyl-D-galactoside, α & β-methyl-D-glucoside, α-methyl-D-mannoside, palatinose, raffinose, salicin, stachyose, sucrose, D-trehalose, turanose, D-xylose	carbohydrates	68% (30/44)
	α & β-cyclodextrin, dextrin, inulin, mannan	polymers	62% (5/8)



a

Figure 3. Standard curve (a=A0562; b=C0661) Comparing turbidity between the Gilford Spectrophotometer and the 96 well plate reader. The CFU and OD values were obtained from the average of n=3 plates and n=8 plates, respectively.



b

Figure 3 (continued).

BIOAVAILABILITY ASSAY (DOC) BY OPTICAL DENSITY

Experiments were performed to measure the change in OD due to the incorporation of DOC into biomass. The OD was measured of bacteria (pure cultures) in a growth medium that consisted of MSM, environmental water containing DOC. The carbon concentrations were 0, 2, and 10 mgC-DOC/l.

There did not appear to be a trend in the growth of the bacteria as carbon concentration was increased. The results were not predictable. Therefore, data is not included. The plots were similar to that as in the section Bioavailability assay (succinate) by optical density (Fig. 4). A sensitivity curve was constructed for the 96 well plate reader and the spectrophotometer (Figs. 3a and 3b). The detection curve for the 96 well plate reader showed that the experiment was setup at 10^4-10^5 CFU/ml.

BIOAVAILABILITY ASSAY (SUCCINATE) BY OPTICAL DENSITY

The environmental isolate, A0562, was grown in 96 well plates containing complete media with varying concentrations of succinate (Fig. 4; Table 6). Succinate was chosen as the carbon source because A0562 was able to metabolize it as determined by the Biolog assay. A0562 grown at 0, 2, and 10 mgC-succinate/l showed little change in OD and their growth curves were at times indistinguishable. This was interpreted as a negative result. A0562 grown at 50 mgC-succinate/l

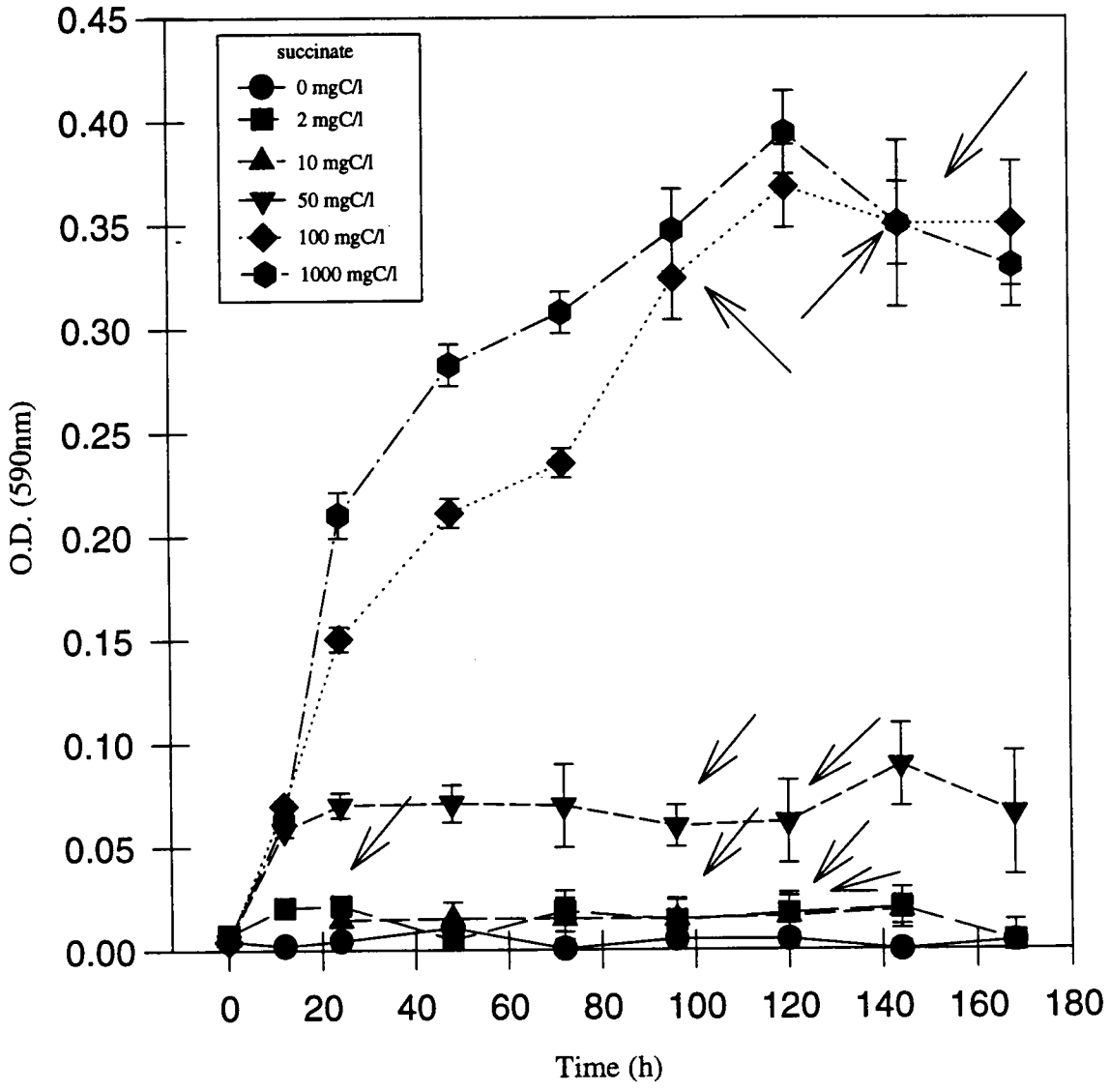


Figure 4. Bioavailability assay (succinate) by optical density. Growth of A0562, subsurface bacterium from Savannah River Site, SC, grown in varying concentrations of succinate (mgC-succinate/l). OD values were obtained from the average of n=96 wells. Arrows indicate when treatments were done (see Tables 1 and 5).

Table 6. Results of bioavailability assay (succinate) by optical density.

succinate concentration (mgC-DOC)/l	Time of additions (h)	Sequential carbon and inorganic nutrient additions	Results (+/-)
0	None	None	-
2	24	MSM (2X)	-
	96	2(mgC-DOC)/l	-
	120	50(mgC-DOC)/l	-
10	120	10(mgC-DOC)/l	-
50	96	MSM (2X)	-
	120	50(mgC-DOC)/l	-
100	96	MSM (2X)	-
	144	MSM (2X); 20ml/l$FeCl_3$; 40ml/l PO_4	-
1000	144	MSM (2X); 20ml/l $FeCl_3$; 40ml/l PO_4	-

reached an OD of 0.07 as did 100 and 1000 mgC-succinate/l. However, the OD of the plate containing 50 mgC-succinate/l had reached a maximum and leveled off. The OD of the plates with the two highest concentrations of succinate continued to increase. The 100 and 1000 mgC-succinate/l had the greatest amount of growth reaching ODs of 0.37 and 0.41, respectively. Treatments to both these plates, only differing in initial concentrations of succinate, were of MSM (2X final concentration) with 20 ml/l $FeCl_3$ and 40 ml/l 5% phosphate.

BIOAVAILABILITY ASSAY (DOC) BY COLONY FORMING UNITS (CFU)

Unfractionated Georgetown surface water at three DOC concentrations (0, 2, and 10 mgC-DOC/l) were setup. The 0 mgC/l served as the negative control and 3-4 orders of magnitude net increase in CFU/ml was routinely observed. The isolate, A0562, shows that cells at all concentrations grew to approximately $1x10^5$ CFU/ml (negative result). A1241 shows a negative result as the cells in 0 mgC/l grew to a higher density than in 2 and 10 mgC/l. The cell numbers of B0612 in 2 mgC/l were within the standard deviation of the negative control. However, the cell numbers of B0612 grown in 10 mgC/l were larger and outside the standard deviation of the negative control. The growth of A1241 shows a break in the 0 mgC/l curve at 96 h. No colonies grew on the agar plate at that time reading. C0571 grew to maximal cell densities of $9.3x10^5$, $2.1x10^6$, and $2.8x10^6$ at 0, 2, and 10 mgC/l, respectively. D0544 was unculturable at all concentrations after the 0h plating. The negative control of G13 grew to $1.8x10^6$. The 2 and 10 mgC/l grew to $3.9x10^6$ and $8.6x10^6$, respectively.

V. DISCUSSION

Subsurface bacteria from the Savannah River site (SRS) differed from surface water bacteria from Georgetown. The subsurface isolates from SRS were mostly G- and metabolized primarily carboxylic acids and amino acids. The surface water isolates from Georgetown were mostly G+ and metabolized primarily carbohydrates. The environment of the two sites are markedly different. Fastidious bacteria probably found subsurface growth a more limiting environment. If their nutritional requirements were not adequately met, they would be in a poor position to compete for dominance. Ecologically, over time, bacteria from a site were dominated by other bacteria (genera) and have either died off or are in very low numbers. If an organism cannot create or find a niche for itself, its survival is in jeopardy.

Pseudomonas and *Acinetobacter* were the most common genera from SRS that were used in these tests. *Bacillus* was the most common genus from Georgetown that was tested. Molecular analysis of bacteria (moles percent G+C) from SRS indicated that 60% of the isolates tested had G+C values similar to those of *Pseudomonas* and 12% had values similar to those of *Acinetobacter* (Jiménez, 1990). An API-NFT rapid test was done by Jiménez (1990) for bacterial identification on isolates from the same site. The isolates studied by Jiménez were not the same as in this study. It was found that bacteria from different geological formations with the same phenotype did not have the same DNA composition suggesting that adaptation had occurred due to the environmental conditions. Thus, lack of DNA relatedness may be cause enough for a different identification.

If the results of the API-NFT and the Biolog assays are compared, both assays show a limitation in their interpretation. The API-NFT assay was performed by David Balkwill (SMCC at Florida State University). Only one environmental isolate from the set used in this study has an API-NFT identification. A0562 was identified as an *Acinetobacter calcoaceticus* var. *lwoffi* and *Acinetobacter* genospecies 13 by API-NFT and Biolog, respectively. A caveat to both these identification systems is that their data was developed from clinical isolates. Thus, unless the tested organisms are similar to a clinical isolates their identification is poor (Holmes, 1994). Clinical isolates tested by the Biolog system (Holmes, 1994) identified 92% of oxidase-positive fermenters (n=64). A similar study consisting of 41 American Type Culture Collection (ATCC) reference taxa and 45 gram-negative isolates from water samples was conducted. Of the 41

ATCC isolates (40) 98% were identified to the correct genus (Klinger, 1994). Forty-five isolates were recovered from a prototype water-recycling system and were identified by Biolog. The Biolog system identified 42 (93%) isolates (Klinger, 1994).

Subsurface bacteria from SRS metabolized primarily the carboxylic acids and amino acids. Surface water bacteria from Georgetown primarily metabolized carbohydrates. The two bacteria that were able to metabolize DOC in Georgetown surface water were B0612 and G13. B0612 was identified as *Klebsiella*. The values for similarity and distance at 24 h, 0.753 and 3.375, respectively, were not as close enough to be considered a good identification (Table 3). G13 was not identified by this assay at either 4 or 24 h. Similarity is not a probability or a goodness of match value. It is a goodness of identification value. At similarity value ≥0.5 at 24 h has an identification accuracy of ≥95% (Barbara So, pers. comm.). An interesting compound that is included in the Biolog GN plate is bromosuccinic acid. Brominated cpmpounds were screened by Biolog Inc. for inclusion in the assay during development of the system. Bromosuccinic acid was selected over other compounds bbecause it provided better identification results (Barry Bochner, pers. comm.).

A drawback of the Biolog assay (version 2) is that although the database is large (260 species or groups from a culture collection of over 5000 strains) most isolates are clinically-derived and it is possible that an environmental isolate being identified is not in the database. However, the Biolog is an excellent assay system for the tentative identification of environmental isolates. A strength of this system is that in order for a cell to metabolize 95 different carbon sources, a plethora of catabolic genes must be activated. Although many genes may have overlapping function, it is a broader profile of gentoypic and phenotypic expression rather than a highly conserved sequence that may have little divergence from different genera (e.g., 16S rRNA). However, one can not rule out other molecular analyses for the identification of environmental bacteria.

Only four isolates from SRS used in this study were examined by 16S rRNA-targeted hybridization probe analysis (B0612, B0719, D0455, and D0544; D. Balkwill, pers. comm.). The probes used for hybridization were *Pseudomonas fluorescens* and related strains, and four different species-specific probes: *Comamonas (Pseudomonas) testeroni, Acinetobacter calcoaceticus, Arthrobacter globiformis, and Pseudomonas (Burkholderia) cepacia.* B0612

probed positive with the species-specific probe for *Arthrobacter globiformis*. D0455 probed positive with the species-specific probe for *Pseudomonas fluorescens* and related strains. B0719 and D0544 did not probe positive with any of the probes tested. Data from the Biolog identification and 16S rRNA probe hybridization were compared. B0612 and D0455 were identified by Biolog as *Klebsiella pneumoniae* and *Corynebacterium jeikeium*, respectively.

STANDARD CURVE/SPECTROPHOTOMETER DETECTION LIMIT

Although the 96 well plate reader could detect cell numbers at ODs as low as 0.03, the data from the Bioavailability of DOC by CFU showed repeatedly that when growth media containing only DOC, MSM, and bacteria at ca. 10^3-10^4 CFU/ml, grew to 10^6-10^7 CFU/ml. Therefore, the OD method for detection of bacterial growth at such low carbon concentrations does not seem to be sensitive enough. According to the sensitivity curve for the 96 well spectrophotometer, we were working at the lowest region of detection with the maximum number of cells sustainable in the given carbon concentration. The lower limit of cell density detection of the 96 well plate reader and Gilford spectrohotometer was determined by stepwise dilutions. Ideally, a doubling of cell number should have shown a similar increase in OD. It is known that spectrophotometers are only sensitive within a range of cell numbers, from 10^6 to 10^9-10^{10} cells/ml, with variations depending upon the bacteria and wavelength (Van Demark, 1986). OD measurements (Gilford) of A0562 showed that OD and CFU's did not increase proportionally until OD 0.003 ($3.3x10^7$ CFU/ml). Similarly, OD measurements (Gilford) of C0661 showed that until OD 0.6 ($1.3x10^5$) the spectrophotometer lacked sensitivity. At cell densities below $1.4x10^7$ CFU/ml (OD 0.003; A0562; Fig. 3a) and $3.2x10^6$ CFU/ml (OD 0.013; C0661; Fig. 3b) the 96 well plate reader lacks sensitivity. It was determined that the experiments for this study were to be setup at OD >0.03 (ca. 10^7 CFU/ml) so that all turbidity readings were above the lower detection limit.

This measurement (detection limit) was a bacterial suspension of known OD and was further diluted. It was not a growth curve. Although the starting OD of A0562 and C0661 with the Gilford and 96 well plate reader were approximately 0.002, the CFU numbers were higher for A0562 (ca. $1.5x10^6$; Fig. 3a). The CFU number for C0661 were ca. $1.0x10^4$ (Fig. 3b). This difference in cell numbers is an example of how rough of an approximation OD can be and the

narrow range of its sensitivity. C0661 illustrates that the 96 well plate reader is not sensitive until an OD of 0.004 reached.

BIOAVAILABILITY ASSAY (DOC AND SUCCINATE) BY OPTICAL DENSITY

Data obtained by this method indicated that bacteria could not grow on the DOC in surface water because the change in OD was not greater than the change from the negative control (0 mgC-DOC/l). The bioavailability assay by OD was done to assess the growth of bacteria in 96 well plates. It was assumed that an increase in OD was due to an an increase in cell density. Also, the data was not reliable as the results were not reproducible. There was a possibility that it was the limitation of inorganic nutrients that was resulting in poor growth. Also, it is interesting to note that the subsurface bacterium, A0562, was isolated from an environment that contained <1 mgC-DOC/l. This bacterium did not grow well until the carbon concentration (succinate) was >50 mgC-succinate/l.

The Bioavailability Assay (succinate) by OD was done to determine if inorganic nutrients were limiting. This assay was similar to the bioavailability assay (DOC). Both measured changes in OD (every 24 h) as an indicator of bacterial growth. The plates were treated (solutions were added) at selected points (Fig. 4; Table 3). After treatments, the OD of the 96 well plates was remeasured to take into account any changes in OD that may have occurred due to addition of solutions. In some plates the OD dropped while in other plates the OD increased. In both cases the changes in OD after the addition of the solutions (treatment) were within the standard deviation of the OD prior to treatment (i.e., there were no changes in OD beyond the range of the standard deviation). Therefore, changes in the OD due to addition of solutions may be disregarded. After treatment, the OD did not increase which indicated that inorganic nutrients were not limiting. Poor growth in the plates containing 0, 2, and 10 mgC-succinate/l may have been due to too low of a carbon concentration to sustain the cell density. This was concluded from the fact that the OD in the plates containing 50, 100 and 1000 mgC-succinate/l showed large increases in the OD.

There is possibility that there may have been carbon limitation or the buildup of metabolic byproducts resulted in growth inhibition. The latter may be ruled out because the 50, 100 and 1000 mgC/l samples showed large increases in OD (Fig. 4). Also, it is unlikely that at such low carbon concentrations (2 and 10 mgC-succinate/l) the bacteria would have generated

enough metabolic waste products since one of the results of starvation is the decrease in metabolic rate (Givsov, 1994). The formation of ultramicrocells is a phenomenon known to occur when bacteria are exposed to starvation conditions (Chesbro, 1990; Kjellberg, 1984; Nyström, 1990). Although, these were not investigated, it would be interesting to find out if the several-fold increase in cell number due to ultramicrocell formation results in a marked change in OD. It was concluded from the bioavailability assay (succinate) by OD (Figs. 3a and 3b) that the inoculum cell density was too high for the supplied carbon. To remedy the problem of high inoculum in a low carbon medium, the bioavailabilty assay by CFU was done.

BIOAVAILABILITY ASSAY (DOC) BY CFU

The subsurface bacteria, A0562 and A1241, (SRS; Figs. 5 and 6) did not grow on natural DOC in surface water from Georgetown, SC and are included to show that not all bacteria grow on natural DOC. They show a typical trend if DOC could not be metabolized. This assay was similar to that of Palumbo (1991) where a low inoculum of bacteria ($2x10^3$ CFU/ml) was added to deep subsurface well water from SRS (wells P24, P28, and P29) containing approximately 0.35mg of carbon per liter. All of the carbon in the water was found as a hydrophobic neutral fraction. The water obtained in the cell numbers reached $1x10^6$ CFU/ml. Palumbo found that surface water (which had the highest carbon concentration) supported more growth than deep well water followed by well water from the near-surface. Also it was found that addition of inorganic nutrients yielded increased bacterial growth. However, the preparation of the initial inoculum was different from this study. In this study, bacteria were lifted off an agar plate and starved for 72 h in complete medium which contained vitamins. It was felt that the addition of inorganic nutrients and vitamins would aid in prolonging respiration and more complete use of carbon stored within the cells. Other researchers have also used the technique of using a low inoculum in low carbon media (Governal, 1992; Pahm, 1993; van der Kooij, 1980).

The bioavailability assay by CFUs solved the problem of having a high inoculum in a low carbon medium. Also, this assay showed selectivity because not all bacteria added to DOC in surface water yielded the same increase in CFU/ml (Figs. 5-8). In some cases, the negative control (0 mgC-DOC/l) reached the same or higher cell density as the 10 mgC-DOC/l (Figs. 5 and 6). This may be because the DOC in the water was not metabolized by these

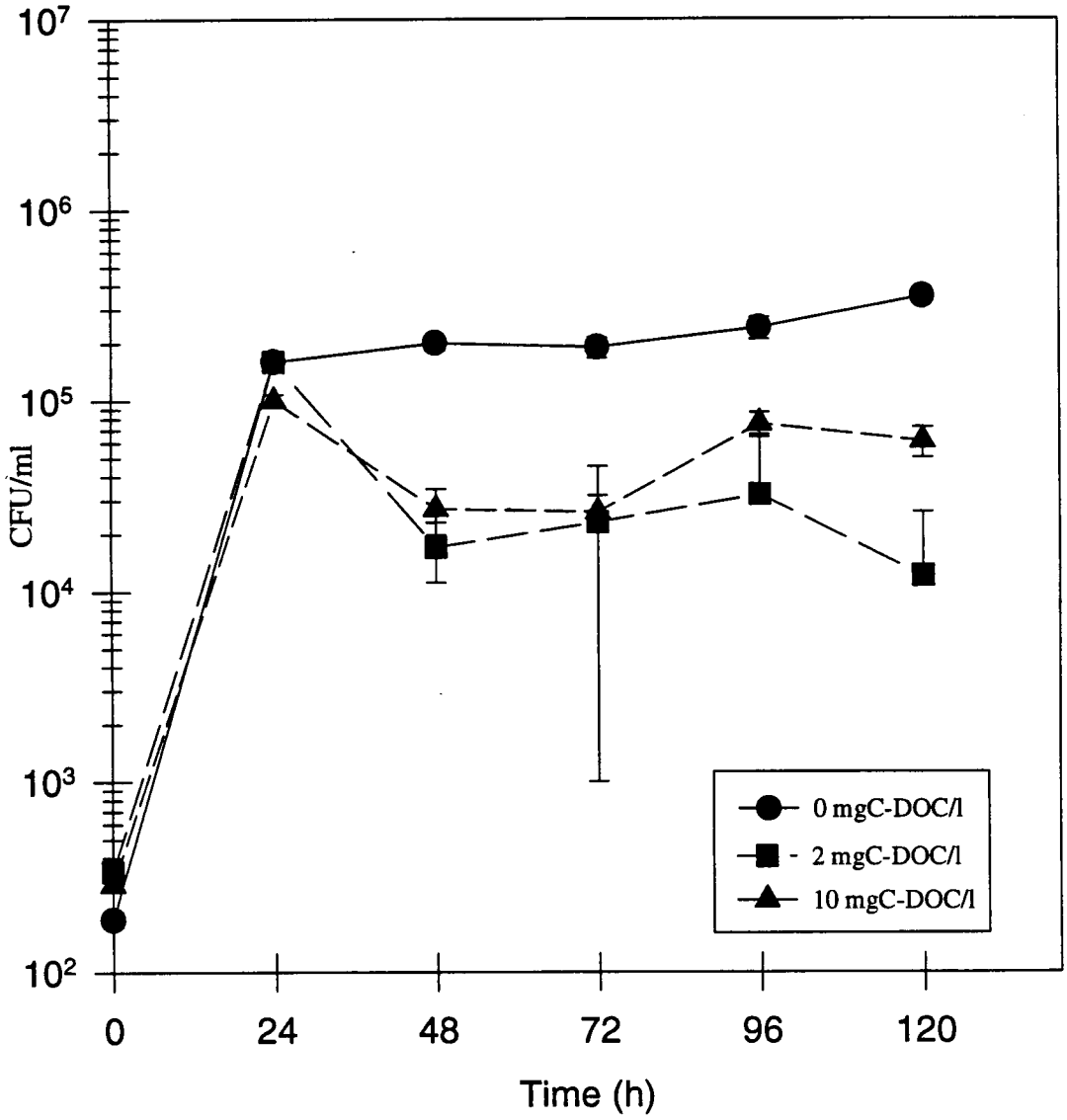


Figure 5. Growth of A0562, subsurface bacteria from Savannah River Site, SC, grown in surface water from Georgetown, SC. The CFU values were obtained fromthe average of n=3 samples.

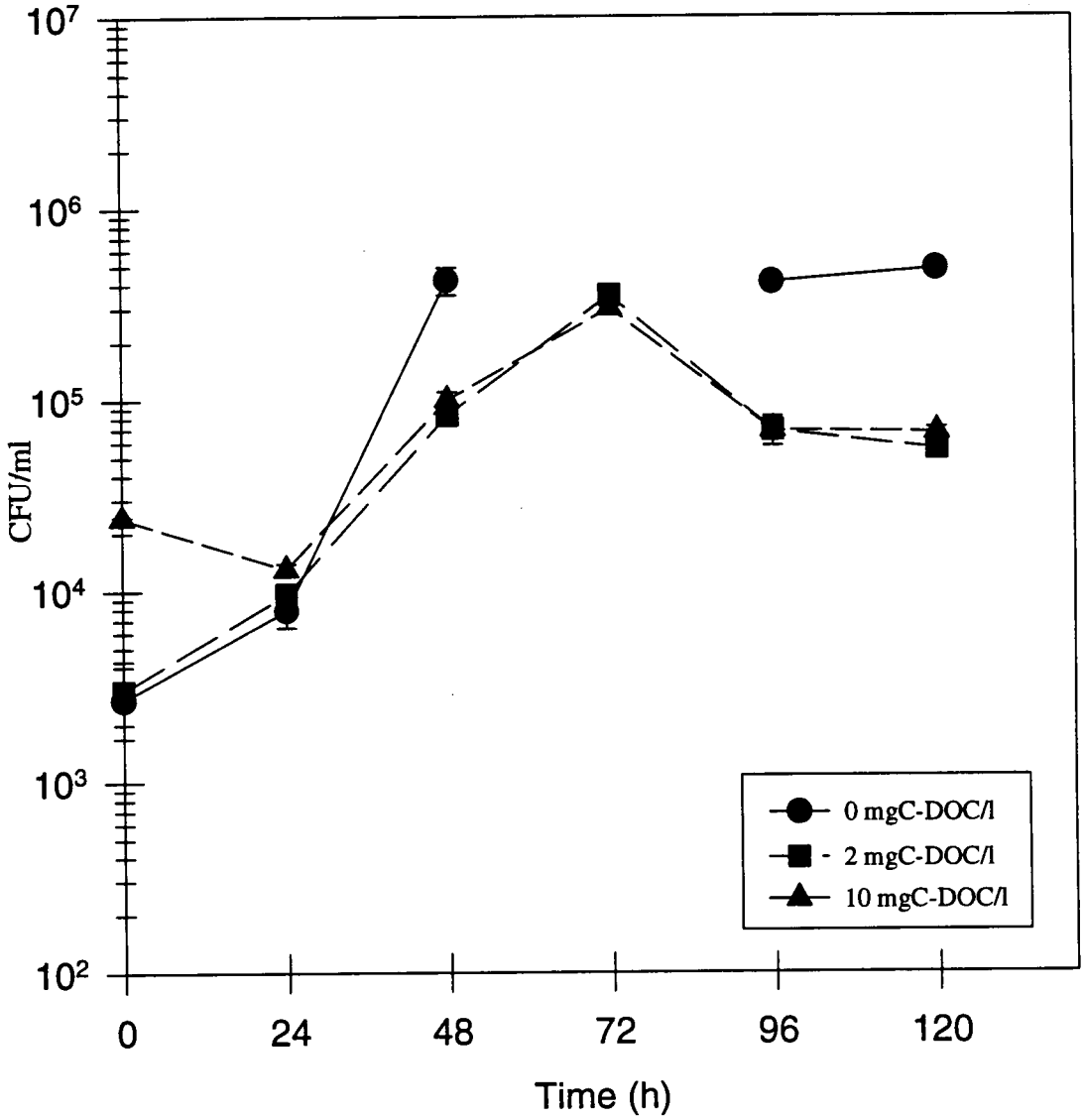


Figure 6. Growth of A1241, subsurface bacteria from Savannah River Site, SC, grown in surface water from Georgetown, SC. The CFU values were obtained from the average of n=3 samples.

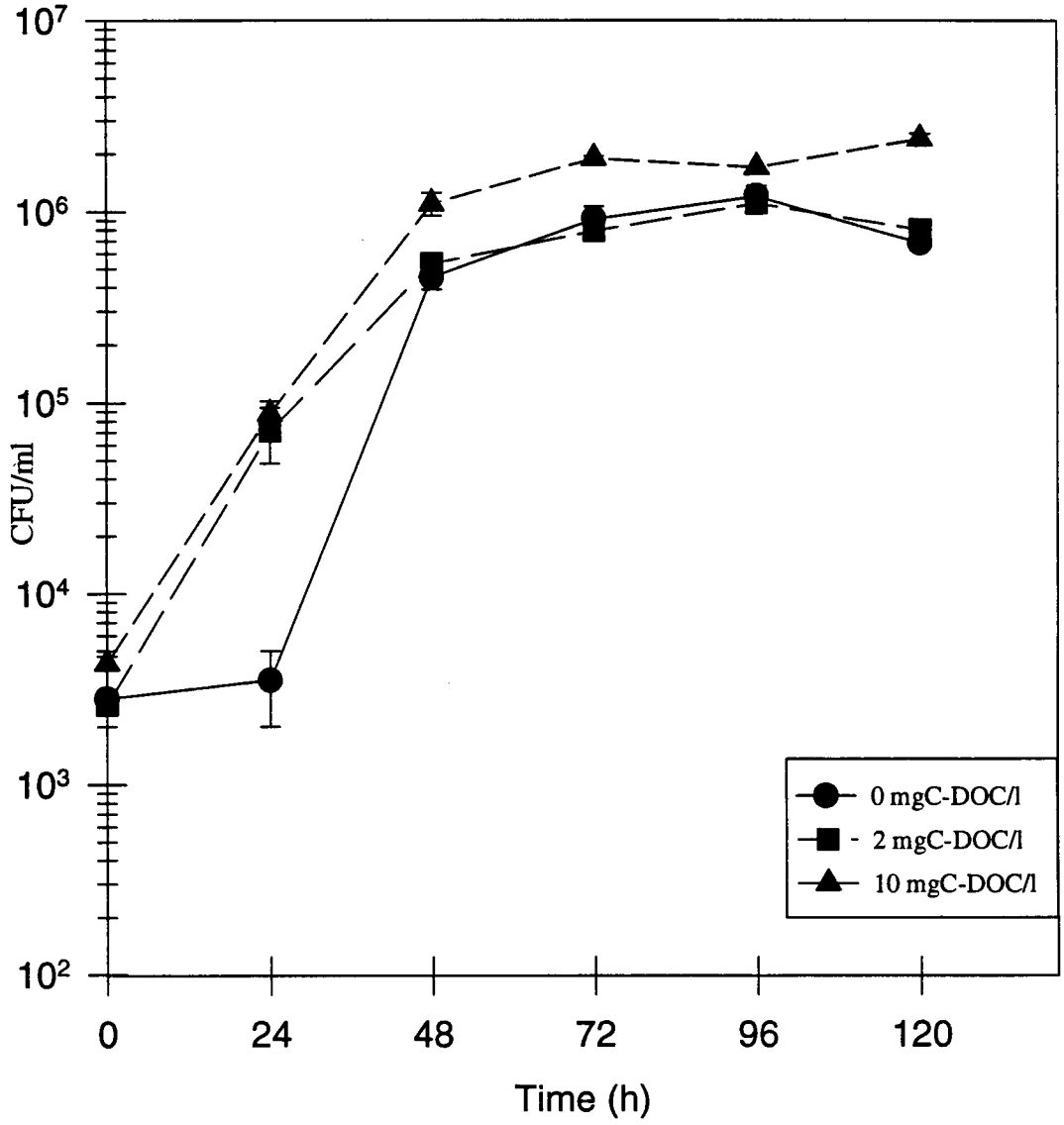


Figure 7. Growth of B0612, subsurface bacteria from Savannah River Site, SC, grown in surface water from Georgetown, SC. The CFU values were obtained from the average of n=3 samples.

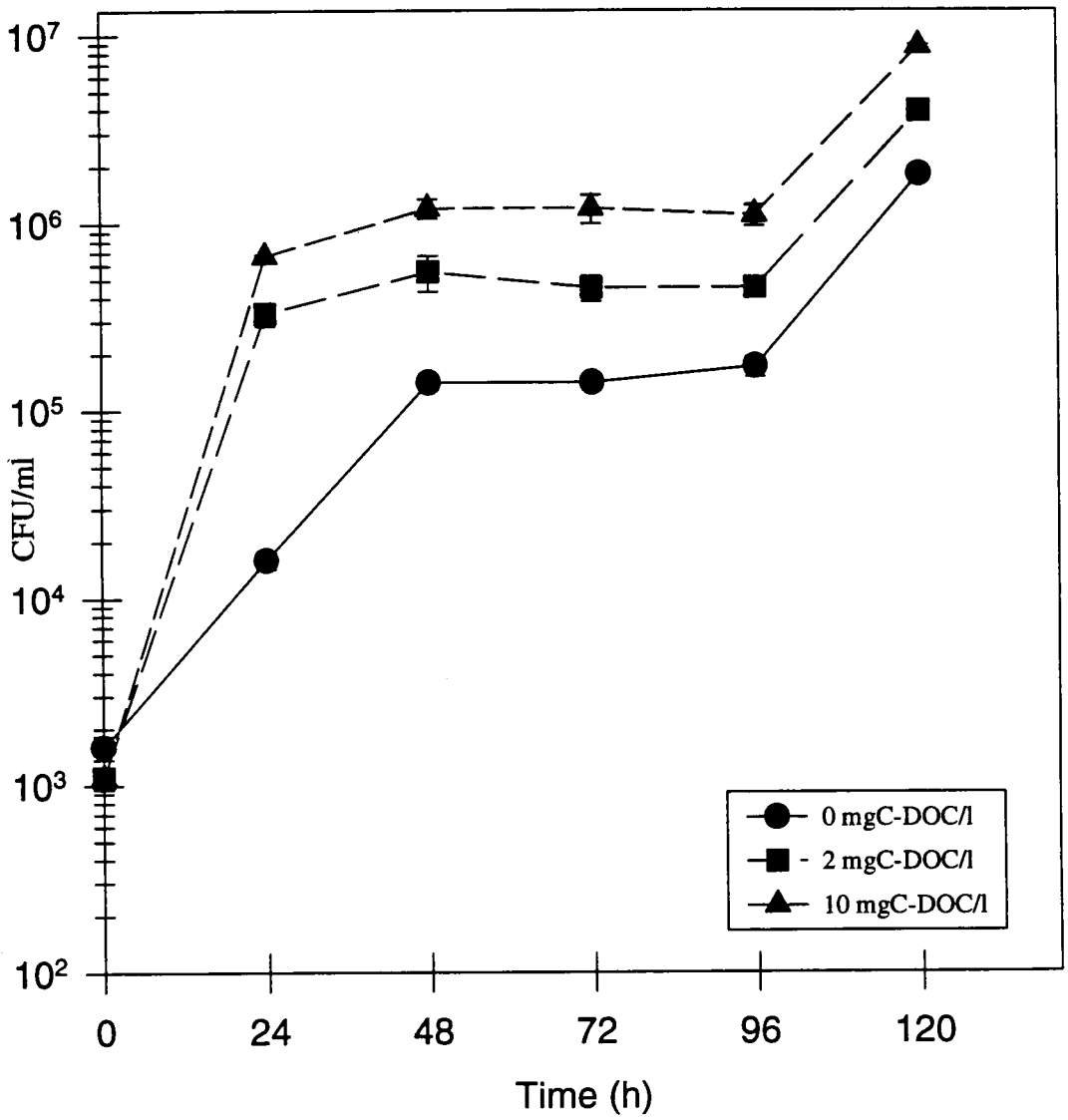


Figure 8. Growth of G13, surface water bacteria from Georgetown, SC grown in surface water from Georgetown, SC. The CFU values were obtained from the average of n=3 samples.

bacteria. Instead, the bacteria in the assay could have been metabolizing the vitamins. Also, the formation of ultramicrocells may have contributed to the increase in cell number.

Ultramicrocells, also known as dwarf, mini, pico-, or nano-bacteria, may appear as vibrio, coccoid, spirilla, and coccobacilli shapes. One of the first researchers to adopt the term ultramicrobacteria was Torrella (1982). The term minibacteria was not used becasuse of the possibility of confusion with minicells of *Eschericia coli*, which are anucleated cells. This decrease in cell size due to starvation is a survival response. This change results in an increased surface to volume ratio and allows for a more efficient substrate scavenging system (Nyström, 1990). Ultramicrocolony is the term used to describe colonies formed by ultramicrocolonies and are not visible to the naked eye. Novitsky (1978) studied Ant-300, a psychrophilic marine *vibrio.* Several notable changes were discovered. It was found that there was a 40% decrease in cellular DNA and protein; the 1 μm x 4 μm rod changed in shape to form a 0.4 μm sphere; the cellular volume was reduced 11-fold; and of the 70% of trichloroacetic acid precipitable total cellular organic carbon indicating that 30% is low molecular weight compounds, there was no carbon pool detected. Despite these changes, a 400-fold increase in cell number was noted. Only 30-40% of bacteria with diameters >0.6 μm, 10% of bacteria between 0.4-0.6 μm, and 0.2% of bacteria <0.4 μm formed colonies (Bakken, 1987). The responses to starvation include fragmentation in to many cells and cell shrinkage. By fragmenting the probability that a nutrient source will be encountered is increased. Secondly, shrinkage increases the surface area to volume ratio and helps the cell escape predation by bacteriovores (Morita, 1988).

Ultramicrocells may most easily be isolated by their ability to pass through a 0.4 μm and or 0.2 μm filter (Hood, 1987). The induction of ultramicrocells may be performed in a manner similar to that of Hood (1987). Bacteria could be nutrient (carbon) deprived by growth in a low carbon medium. After 120 h, cells could be filtered through 0.2 and 0.4 μm filter. Bacteria may be enumerated by plate counts, most probable number and acridine orange direct counts. The most appropriate source of carbon in such experiments would be aquatic humic substances. Thus, the well-known phenomenon of ultramicrocell formation that may have been contributing to the increase in cell numbers, especially in the 0 mgC-DOC/l, may be determined.

Although aquatic humic substances make up 50% of the dissolved organic matter, a majority of carbon found in lake water (>60%) is inaccessible to most bacteria (Amy, 1992; Geller, 1983, 1986; Meyer, 1987) Geller (1985) reported that the labile low molecular weight

substances combine with the higher molecular weight substances and promote humification forming increasingly recalcitrant compounds. The labile low molecular weight substances combine with the more recalcitrant higher molecular weight substances, promote humification, and form increasingly recalcitrant compounds.

Bacteria, inoculated at 10^2-10^3 CFU/ml, consistently grew to ca. 10^6 CFU/ml in environmental water containing 10 mgC-DOC/l. Additionally this assay shows that the maximum number of cells 10 mgC-DOC/l can support is approximately 10^6-10^7 CFU/ml. This further supports the fact that if the assay for bioavailability (DOC) by OD was setup at 10^6 CFU/ml, a change in cell density would be indistinguishable. The bacteria that could metabolize DOC in surface water were B0612, and G13 (Figs. 7-8). Note that one growth produced from data by this assay show that the curve is broken (Fig.6). They lack CFU numbers at 72 h. The bacteria did not grow and it cannot be explained because because all other time readings had CFU numbers. It may be that sample was overlooked and not plated. If one compares the OD assay with the CFU assay, the latter is more sensitive of the two.

Not all bacteria grew in this medium. Reasons for this include that the medium was not optimized for any bacteria. It is possible that components in the medium may be toxic to the bacteria. In the environment bacteria probably do not see concentrations of vitamins and minerals as high as in this study. Also, interactions of compounds in the medium with the DOC in the water could have made carbon uptake or metabolism difficult.

This study has shown that the DOC found in Georgetown surface water can be metabolized. It could not be determined from this study if the DOC in the surface water was mineralized, the extent of mineralization or the type/fraction of DOC that was mineralized. Other experiments that would logically follow are testing DOC in groundwater, fractionated and size separated (by molecular weight) DOC in surface/groundwater.

A potential flaw with this study was that the environmental isolates were subcultured on nutrient agar plates. After several subcultures the number of mutations (variants) increase (Gherna, 1981). A remedy to this is that only a limited number of subcultures should be done on nutrient agar. Frozen stocks should be maintained at -20°C in an ordinary freezer as a means of storage of cultures. Bacteria can be maintained by this method from 6 months to 2 years depending on the bacteria.

VI. CONCLUSION

The purpose of this study was to compare heterotrophic, aerobic bacteria from the deep subsurface and from surface water for their ability to utilize naturally occurring DOC as a source of carbon and energy. An assay measuring the change in cell number of bacteria in a minimal media supplemented with surface water from Georgetown, SC containing DOC was used. Selected subsurface and surface bacteria from two uncontaminated sites were studied.

It was found that one subsurface soil bacterium and one surface water bacterium were able to metabolize the DOC in surface water from the Savannah River Site. The metabolic profiles indicated that the subsurface bacteria metabolized primarily carboxylic acids and amino acids. The surface water bacteria primarily metabolized carbohydrates.

The Biolog assay was done on the bacteria. The hypothesis was that the more substrates an organism was able to metabolize, the easier it would be able to use DOC as a carbon and energy source. However there appearee to be no correlation between the two. The subsurface soil bacterium, B0612, metabolized 94 substrates whereas G13 metabolized only 21 substrates. Although there were 9 other bacteria that metabolized more substrates than G13, they did not grow on DOC in surface water. The Biolog assay also provided an identification. Most of the bacteria from the subsurface were G- and identified as *Pseudomonas* or *Acinetobacter*. Most of the bacteria from surface water were G+ and identified as *Bacillus* or *Corynebacterium*. API and Biolog identification methods yielded similar results. 16S rRNA probe hybridization results were not in agreement.

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APPENDICES

APPENDIX A

5% phosphate buffer solution (pH 6)

$Na_2HPO_4 \cdot H_2O$	24.5 g
$NaH_2PO_4 \cdot H_2O$	25.5 g
Distilled water	1.0 l

Balch's vitamin solution (100x; Cote, 1994)

p-Aminobenzoic acid	5.0 mg
Folic acid	2.0 mg
Biotin	2.0 mg
Nicotinic acid	5.0 mg
Calcium pantothenate	5.0 mg
Riboflavin	5.0 mg
Thiamine HCl	5.0 mg
Pyridoxine (vitamin B_6)	10.0 mg
Cyanocobalamin (vitamin B_{12})	0.1 mg
Thiotic acid (lipoic acid)	5.0 mg
Distilled water	1.0 l

Trace elements solution (1000x)

$CuSO_4 \cdot 5H_2O$	50.0 mg
$MnSO_4 \cdot H_2O$	1.0 mg
$Zn(NO_3)_2 \cdot 6H_2O$	70.0 mg
MoO_3	10.0 mg
$CoCl_2 \cdot 6H_2O$	10.0 mg

Minimal salts media (MSM; 10X; pH 6.8; Little, 1988)

$MgSO_4 \cdot 7H_2O$	10.0 g
$CaCl_2$	2.0 g
KNO_3	10.0 g
NH_4Cl	1.0 g
Trace elements solution	10.0 ml

Complete media (20 ml)
autoclaved Milli-Q water
2X Minimal salts media (1X final concentration)
0.4 ml 0.027% $FeCl_3$
0.2 ml Balch's vitamin solution (1X final concentration)

APPENDIX B

Substrates found in a Biolog Gram positive (GP) plate.

carbohydrates (n=44)	N-acetylglucosamine, N-acetylmannosamine, amygadalin, L-arabinose, D-arabitol, arbutin, cellobiose, D-fructose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-gluconic acid, α-D-glucose, m-inositol, α-D-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, melezitose, 3 methylglucose, α-methyl-D-glucosidase, β-methyl-D-glucoside, α-methyl-D-mannoside, palatinose, D-psicose, D-raffinose, L-rhamnose, D-ribose, salicin, sedoheptulosan, D-sorbitol, stachyose, sucrose, D-tagatose, D-trehalose, turanose, xylitol, D-xylose
carboxylic acids (n=13)	acetic acid, α–hydroxybutyric acid, β– hydroxybutyric acid, γ– hydroxybutyric acid, p-hydroxyphenylacetic acid, α–ketoglutaric acid, α–ketovaleric acid, D,L-lactic acid, malic acid, propionic acid, pyruvic acid, succinic acid
amino acids (n=10)	D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine
esters (n=3)	methylsuccinate, methyl pyruvate, D-lactic acid methyl ester
polymers (n=8)	α–cyclodextrin, β–cyclodextrin, dextrin, glycogen, inulin, mannan, tween 40, tween 80
alcohols (n=2)	2,3-butanediol, glycerol
amides (n=3)	alaninamide, lactamide, succinamic acid
phosphorylated chemicals (n=7)	adenosine-5'-monophosphate, thymidine-5'monophosphate, uridine-5'-monophosphate, fructose-6-phosphate, glucose-1-phosphate, glucose-6-phosphate, D,L-α–glycerol phosphate
aromatics (n=5)	adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine

Substrates found in a Biolog Gram negative (GN) plate.

carbohydrates (n=28)	N-acetylgalactosamine, N-acetylglucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, *i*-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, m-inositol, α-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, β-methylglucoside, psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol
carboxylic acids (n=24)	acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α–hydroxybutyric acid, β– hydroxybutyric acid, γ–hydroxybutyric acid, p-hydroxyphenylacetic acid, itaconic acid, α–ketobutyric acid, α–ketoglutaric acid, α–ketovaleric acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid
amino acids (n=20)	D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, D,L-carnitine, γ–aminobutryic acid
esters (n=2)	mono-methylsuccinate, methyl pyruvate
polymers (n=5)	glycogen, α–cyclodextrin, dextrin, tween 40, tween 80
alcohols (n=2)	2,3-butanediol, glycerol
amides (n=3)	succinamic acid, glucuronamide, alaninamide
phosphorylated chemicals (n=3)	D,L-α–glycerol phosphate, glucose-1-phosphate, glucose-6-phosphate
aromatics (n=4)	inosine, urocanic acid, thymidine, uridine
brominated chemicals (n=1)	bromosuccinic acid
GN Plate amines (n=3)	phenylethylamine, 2-aminoethanol, putrescine

VITA

Born June 1, 1967 in Bombay, India. The author briefly attended St. Andrews Elementary before being uprooted at the age of seven. His family landed in Forest Park, Illinois and through the years continued to move eastward. Being obligated to follow, as he was too young to obtain a work permit, his family after a few moves ended up in Rochester, NY-graduated from Brockport High, 1985. He joined the National Guard and finally earned a commission through the Empire State Military Academy, 1991. He received an A.A.S in Chemistry from Dutchess Community College after yet another move and a B.S. in Biotechnology from Rochester Institute of Technology, 1991. While attending RIT, he obtained a co-op position at Bristol-Myers Company and gained a love for science which spurred his interest in graduate school.

He enrolled in the University of Tennessee in the Fall of 1991. His graduate career started at the Oak Ridge National Laboratory (Jan. 1992-Sep 1994) under Dr. Anthony V. Palumbo and ended in the laboratory of Dr. Gary S. Sayler (Aug. 1995). He received his M.S. in Environmental Toxicology in December 1995.

His future plans besides working in industry include the continuation of brewing, running, repairing cars, and the Guard.