Response of Indigenous Heterotrophic Groundwater Bacteria to Low Organic Substrate Availability

by

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(ABSTRACT)

Groundwater is one of the least studied environments, yet many people rely on groundwater for their sole drinking water supply. Little is known about the indigenous microflora, but it is believed to be similar to oceanic planktobacteria due to the low nutrient concentrations occurring in both ecosystems. That is, groundwater microorganisms are atypically small, mostly Gram-negative cells. Also like the oceanic planktobacteria, they may have no affinity for surface attachment and may rely on dissolved low molecular weight organic substrates in dilute solution for their nutrition. Periods of metabolic dormancy may occur when natural substrate concentrations drop below the level required to sustain vegetative cell function. In these studies total cells present were determined by 4'6-diamidino-2-phenylindole (DAPI) epifluorescent counts. The percentage of those bacteria which were metabolically active was determined by a modification of the 2-(p-iodophenyl)-3-(p- nitrophenyl)-5-phenyl tetrazolium chloride (INT) reduction method. Advantages of this method over others include more specific fluorochrome staining, ease of transfer of the cells to the slide, time saved, and ease of microscopic

viewing. Heterotrophic uptake of aspartate, succinate, glucose and fructose by indigenous bacteria was measured and calculations of maximum uptake velocity (V_{max}) and a constant ($K_t + S_n$) equalling the natural substrate concentration (S_n) plus the half-saturation concentration (K_t) were made based on net assimilation (cellular retention) of radiolabeled substrate. Total counts by DAPI staining were 4-12.1 x 10^4 cells/ml of which 17.4 to 20.85% were metabolically active (INT+). Mean maximum uptake velocities ranged from 1.73 to 2000 nmol/l/hr with aspartate being taken up at the highest rate followed by fructose, succinate and glucose.

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INTRODUCTION

Groundwater communities are little studied aquatic environments, and little is known about the indigenous groundwater organisms and their activities (4,16,27). Because almost one-half of the United States population and one-third of the Virginia population relies on groundwater for their drinking water supply (24,26), this aquatic system warrants study and any necessary steps to prevent contamination and to clean up sites presently contaminated. All this necessitates that certain baseline information be obtained. Groundwater habitats often contain low concentrations of organic nutrients, and this characteristic undoubtly influences the heterotrophic microorganisms which are indigenous to these habitats. Although the necessity for adaptations to low organic nutrient seems obvious, such adaptations have hardly been explored.

The scant data available suggest that the groundwater environment, with its paucity of dissolved organic matter, supports a surprisingly large community (10^6 cells/ml) of small (< 0.6 μm), Gram-negative bacteria which have little propensity for surface attachment and which utilize low molecular weight organic substrates for their nutrition (15). Their small size has been attributed to nutrient stress resulting from the low natural substrate concentrations. Another consequence of low available substrate is dormancy which alternates with periods of adequate nutrition. Many heterotrophic uptake studies have been performed to quantify

the rates at which the oceanic planktobacteria utilize substrates (10,14,28,30,34). However, similar studies of groundwater heterotrophic communities have not been accomplished.

This work applies experimental methods comparable to those for oceanic systems to groundwaters to assess the numbers of total and viable bacteria present and to measure the response of these microorganisms to additions of four substrates at low ambient concentrations. It was hypothesized that the groundwater communities would respond similarly to those of oceanic waters.

Four Virginia wells, two in the West Central (Piedmont) area and two in the more organic nutrient rich Coastal Plain, were selected. These well waters had been tested periodically by the Virginia State Water Control Board and established as unpolluted. Representative chemical data on each well are in Appendix A. Data from Parker (unpublished) suggest that the wells contain indigenous microbial communities.

Total cell numbers were determined by 4'6-diamidino-2-phenylindole (DAPI) epifluorescence (20). A modification of the 2-(p-iodophenyl) -3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) method (35) was developed to assess the total viable cells. Heterotrophic uptake experiments, similar to those used on oceanic waters, were performed and maximum uptake velocity (V_{max}) and a constant ($K_t + S_n$) equal to the sum of the natural substrate concentration (S_n) and the half-saturation concentration (K_t) were calculated. These studies may provide a basis for

further work on groundwater pollution, and particularly in the biorestoration of contaminated wells.

CHAPTER ONE--LITERATURE REVIEW

Groundwaters have been relatively little studied despite their recognized importance as aquatic resources. As with surface waters, the chemical, biochemical and bacteriological processes occurring in groundwaters play key roles in their quality. Two fundamental questions yet to be answered are: (i) What microorganisms are indigenous to groundwater, and (ii) what roles do these indigenous microorganisms play? The low concentrations of organic matter in groundwaters suggest a parallel between the nutritional quality of groundwater and oceanic waters. This chapter provides a review of literature pertinent to all studies presented here.

A common finding in aquatic ecosystems is that low growth rates are associated with low nutrient concentrations (4). In many cases, organisms become nutrient-stressed, which results in metabolic dormancy of some cells (15). Stevenson has identified the ability to compete for organic substrates as one of the "fitness traits" for aquatic bacteria (25). In other cases, cell division may continue without concomitant cell growth, resulting in dwarf cells. Upon addition of nutrients to the system, these dwarf cells can regain their "normal" size. It is widely accepted that bacterial cells in many natural systems are smaller than normal (25). The common discrepancy between high numbers of cells as estimated by direct counts and low numbers of viable cells as estimated by any of numerous counting methods is thought to indicate that a large part of the

community is dormant (15). These are termed "viable but non-culturable" organisms, which by definition are alive but do not or cannot divide in or on agar media (22).

The low growth rate of naturally occurring aquatic organisms presents several logistical problems in the laboratory. One of these is associated with the practice of plating and counting colonies on agar media. Only a portion of the cells will grow on these media, and a representative proportion requires relatively complex media at unnaturally high nutrient concentrations be used. Such high nutrient concentrations select for organisms that may be unimportant in the aquatic community, and thus the organisms most indigenous to that habitat are often undetected (15). An alternative to plating is continuous culture in a chemostat, but often microbial growth rates at natural substrate concentrations are too slow for the bacteria to reproduce successfully in such steady state cultures. Even the lowest dilution rate may be too rapid to match generation times under low nutrient conditions (15). This is indicated by a two-stage heterotrophic uptake phenomenon in which uptake occurs immediately and again after 20-36 hours. The lag time is sufficient for activation of dormant cells but not for multiplication (25); generation times in various aquatic systems have been estimated at 20-200 hours with a mean value of 53 hours (22).

In the marine oceanic system, two discrete communities of Gram-negative heterotrophic bacterial rods have been identified. One community consists of cells that are relatively large, live attached to solid surfaces,

and can be plated on agar media. The other community consists of smaller cells that are unattached to solid surfaces, incapable of forming colonies on agar media, and utilize organic substrates from dilute solutions. Cells of the first community are 0.8 to 1.0 μm long whereas those of the second are 0.2 to 0.8 μm long (22). Numerous methods exist for enumerating total and viable bacteria (22), several of which are discussed below.

Radiolabeling and autoradiographic techniques, especially in combination with methods for total cell counts, have been used to enumerate viable bacteria in aquatic samples. For example, autoradiography combined with acridine orange direct counts (AODC) indicated that between 2.3 and 56.2% of the bacteria in Kiel Bight were actually metabolizing (18). The technique required several days and careful pH control during the staining steps, which made it somewhat complicated.

Two other methods that have been employed to enumerate viable bacteria are the nalidixic acid method (17) and the microcolony formation technique (8). The nalidixic acid method results in elongation of viable Gram-negative cells due to the inhibition of cell division by the nalidixic acid, while the other technique results in microcolony formation in slide cultures. In a study of several freshwater environments, 4.3 to 9.7% of bacteria were found viable with the nalidixic acid method; 4.8 to 14.7% of the same community were found to be viable with the INT procedure which will be discused below (17). The drawback to the nalidixic acid method is its effectiveness only for Gram-negative bacteria. Although most ma-

rine and groundwater bacteria are Gram-negative, this method alone is still inadequate. With respect to microcolony formation, estimates of the viability of planktonic bacteria in several freshwater samples ranged from 18.4 to 48.7% (8). This method required a great deal of careful preparation and had a long completion time. Furthermore one might question whether true bacterioplankton are all capable of colony development on surfaces and such media.

Direct counts using fluorochrome stains represent another approach for the enumeration of aquatic bacteria. Total cells present in marine waters as estimated with acridine orange (AO) are 10^4-10^6 cells per ml (23). This number reflects total cell numbers but indicates nothing about the viability of the organisms. AO fluoresces red in cells with a high RNA:DNA ratio, implying active cells; it fluoreses green where a low RNA:DNA ratio is present, implying inactive cells. However, several other factors including the pH of the fluorochrome, the stain incubation time, and the concentration and age of the stain solution can affect the fluorescent color (22). In addition, AO complexes with some particulate debris, resulting in enumeration errors. This reduced reliability of AODC alone to determine viability has led to the combination of AODC with the reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) which, when acted upon by dehyrogenases, produces red intracellular formazan deposits that are visible by brightfield microscopy. This combined method, called AOINT, has indicated that 5 to 36% of the AO staining microorganisms in surface freshwaters are INT+ and

that 6 to 12% are INT+ in Baltic Sea coastal waters (35). One problem with this method is that the formazan byproduct is soluble in immersion oil, in which fluorochrome-stained preparations are commonly mounted for microscopic observation. An alternative is to mount the cells between two layers of gelatin (26), which prevents the dissolution of the formazan in the immersion oil. Another procedure involves staining with malachite green instead of a fluorochrome for determining total cell numbers after INT reduction has occurred; however, the relatively non-specific malachite green stains debris and dead cells. This method, termed MINT, has been applied mainly to filamentous bacteria.

Fluorochromes other than AO, such as ethidium bromide, fluorescein diacetate, mithramycin, and 4'6-diamidino-2-phenylindole (DAPI), can also be used for direct enumeration counts. DAPI has been successfully used in aquatic microbiology (20) although it has been reported to be unsuccessful when used in combination with INT reduction (26). DAPI fluoresces blue and complexes with double-stranded DNA, indicating an intact, possibly functional genome (20). It does not complex with particulate matter or RNA as does acridine orange and, due to its specificity for double-stranded DNA, it should not stain non-living organisms, the nucleic acids of which would quickly be degraded upon cell death. In addition, the fluorescent glow that is created allows the detection of cells below the limit of resolution of the light microscope although the cell morphology cannot be determined.

It is useful to study nutrient uptake by the microorganisms after they have been enumerated. Techniques to study heterotrophic uptake in aquatic systems based on incorporation of radiolabeled substrates began to be developed in the late 1950's. In 1962 Parsons and Strickland combined heterotrophic uptake experiments with Michaelis-Menten enzyme kinetic calculations (34). Many elaborations on their work have followed, including the introduction of the use of substrate concentrations approaching those considered natural (34). Subsequent studies have expanded on these basic experiments. In a study of lake bacterial uptake of acetate and glucose, Wright and Hobbie (34) identified two distinct uptake mechanisms. One was simple diffusion, but the other appeared to be active transport resulting in a half-saturation concentration (K_m) of 1×10^{-7} mol/l for acetate and 4×10^{-8} mol/l for glucose. These K_m values suggest high substrate affinities, because substrate affinity is inversely proportional to the magnitude of the K_m value. In a comparison of heterotrophic uptake of five substrates by marine bacteria, Iturriga and Zsolnay (14) found that glutamic acid had the highest maximum uptake velocity (V_{max}), followed by arginine, glycine, glucose, and glycolic acid. In a study of uptake of an amino acid mixture Ferguson and Sunda (7) obtained half-saturation concentration plus natural substrate concentration ($K_t + S_n$) values of 0.18 to 7.1 nmol/l for surface ocean waters (6) but the values decreased with sample depth. In these studies, kinetic parameters had higher values in samples containing higher numbers of bacterial cells.

According to Bell (2), most natural waters contain concentrations of total organic carbon that are usually in the range of 1 to 6 mg C/l. Accordingly, oligotrophic bacteria have been defined as those which can grow on media containing 1-15 mg organic C/l. Two functional groups of microorganisms were identified in the oligotrophic waters of Lake Biwa, namely, obligate oligotrophs which could not grow in nutritionally rich media and facultative oligotrophs which can withstand a wider range of substrate concentrations (13). The K_m values of facultative oligotrophs were dependent on the substrate concentration present, increasing with greater nutrient levels. These microorganisms displayed more efficient uptake when low nutrient conditions existed, perhaps indicating high substrate affinities.

A specific activity index (SAI) was developed by Wright for the comparison of uptake on a per cell basis (33). Three different indices were described based on maximum velocity, turnover rate, and direct uptake; the parameter selected is divided by the total direct counts, resulting in a per cell basis for comparison of different samples. The SAI has been used as an indicator of the physiological state of the bacteria, i.e., in determining whether cells are in a state of dormancy.

Very few studies have addressed groundwaters and most researchers have studied groundwater-saturated sediments. Most have emphasized plate and epifluorescent total counts. Balkwill and Ghiorse (1) have studied various groundwater-saturated sediment samples. In one study AODC values were 2.9-9.8 x 10^6 cells per gram dry weight of sediment material. Fur-

ther study of these same samples yielded a wide range of colony forming units (CFU); values obtained were from 6.3 x 10^2 to 6.5 x 10^6 CFU/g dry material, and the bacteria grew better on dilute media. Of the isolates, 85-90% were Gram-positive "coccoid rods" 0.4 to 0.9 μm in length; no eucaryotes were isolated. A similar study by the same investigators (9) showed AODC values in saturated sediments to be 1-10 x 10^6 per gram of dry material; however, less than 100 CFU/g dry material were obtained. Again, no eucaryotes were isolated. Values of 3.4-9.8 x 10^6 cells per gram dry material have been reported for other groundwater-saturated sediments (31). Nearly 50% of the cells were viable based on CFU, but plate counts varied 100-fold when various media were used. Most isolates were small (< 1 μm) Gram-positive coccoid forms. No variation in numbers occurred with depth, and attempts to stimulate bacterial degradation of 1,2-dichloroethane, 1,1,2-trichloroethane, trichloroethylene, and tetrachloroethylene were unsuccessful (31). Another survey of saturated sediments determined microbial biomass by five methods (29). Measurements of phospholipids, phospholipid ester-linked fatty acids, muramic acid, hydroxy fatty acids of lipopolysaccharide (LPS) of Gram-negative cells and ribitol in Gram-positive cells all resulted in approximately equivalent values of 10^7 bacteria per gram dry weight. ATP assays have also been performed on groundwater-saturated sediments in cores, yielding values of 0.37 to 0.74 ng/g ATP (28). Accompanying AODC values were 6.2-10.75 x 10^6 cells/g but only 1-10% of the cells were capable of producing colonies on agar. The ATP measurements were made with a new method these researchers developed in an effort to obtain a reliable

measure of microbiological activity in underground waters. AOINT was attempted in these experiments but was deemed "weakly positive" and not pursued further (28).

In free-flowing groundwater systems bacteria have been enumerated on R2A and SMA media; the values were 310 CFU/ml and 118 CFU/ml, respectively (24). Eighty-nine percent of all isolates were Gram-negative although the number of genera was limited. Other free-flowing groundwater systems have yielded AODC values of 3.33-14.3 x 10^4 cells/ml, although plate counts varied widely with the nutrient concentration (4). Isolates were predominantly Gram-negative rods except during maximum volume flow when Gram-positive rods dominated. Various methods to determine viability were employed; however, no results were obtained from AOINT, MINT, or slide culture techniques. The nalidixic acid method estimated that 2.64 to 40.4% of the cells were viable (4). AODC values for another groundwater system averaged 10^5 cells/ml with increases up to 10^6 when the soil surrounding the aquifer thawed (16). Forty percent of the isolates were Gram-positive and no strict anaerobes were isolated. Mineralization of ^{14}C-cinnamic acid and ^{14}C-lignin-labeled lignocellulose was also studied. Both compounds were partially degraded, and cinnamic acid mineralization was eight times more efficient than that of lignocellulose.

Only one study has been made of heterotrophic uptake by indigenous groundwater bacteria. Ventullo and Ladd (27) surveyed five aerobic groundwaters in Ohio and Ontario. Samples were taken at shallow depths

from gravel and clay aquifers in which the ambient temperature was approximately 10° C. The Canadian wells were subject to contamination by septic tank drainage. After obtaining AO counts and plate counts on dilute media, Ventullo and Ladd determined most probable numbers (MPN) of bacteria capable of metabolizing specific substrates. In these experiments ^{14}C-labeled substrates at concentrations of 1 to 10 $\mu g/l$ were added to groundwater samples and incubated without agitation in the dark at 15° C for 4-5 weeks. For an unexplained reason, the agar plates were incubated at 19° C and the MPN tubes were incubated at 15° C although the ambient groundwater temperature was 10° C. Both assimilation and respiration were measured in the MPN experiments. In addition, heterotrophic uptake (both assimilation and respiration) of D-glucose, L-arginine, L-glutamate, and an L-amino acid mixture was measured by the turnover time-tracer method. With this technique, substrate was added at only one concentration but samples are incubated for various lengths of time. Substrates were added at concentrations of 0.2 to 1.3 $\mu g/l$, depending on the substrate. Attempts were also made to measure both long-term and short-term biodegradation of synthetic compounds such as sodium nitrilotriacetate, 2,4-dichlorophenoxyacetate (2,4-D) and benzoate. AODC values ranged from 0.036-1.21 x 10^6 cells/ml and plate counts varied from 0.31-8.6 x 10^3 CFU/ml. MPN values fell between the AODC and plate counts, in the range of 0.2-4.0 x 10^4/ml. Heterotrophic uptake activity was recorded as turnover time and ranged from 100 to 2048 hours depending on the substrate. All substrates except 2,4-D were degraded with Vmax values ranging from 48-1405 ng/l/hr, depending on the substrate (27).

Overall, the paucity of data on heterotrophic uptake in groundwater stands out as a large void in aquatic microbiology. A need still exists for methods specific for the special conditions of subterranean ecosystems.

CHAPTER TWO--ENUMERATION OF HETEROTROPHIC GROUNDWATER BACTERIA

Introduction

A first task in studying a bacterial community is to enumerate and characterize its constituents. In the case of groundwater, several researchers have enumerated the indigenous bacterial communities by various methods. The AODC method has been used on both groundwater (4,24) and sediment (1,9,28,31) samples. Plate counts on dilute and enriched media have been compared with direct counts to determine the percentage of viable cells (1,4,9,24,31). Other enumeration techniques have been employed such as the nalidixic acid method (4) and ATP assay (28). Buchanan-Mappin et al. (4) also attempted the AOINT, MINT, and slide culture techniques without success. White et al. (29) did an extensive biomass study in which five different cellular components were assayed. However, few attempts have been made at characterizing groundwater isolates. The published results indicate that Gram-negative rods predominate in free-flowing groundwater bacterial communities (4,24).

The bacterial populations in the groundwaters studied were enumerated by DAPI epifluorescent counts and INT-DAPI-FTF counts. CFU were determined by plate counts on a dilute medium, and some isolates were characterized.

Materials and Methods

Four groundwaters were sampled, two in the Coastal Plain (CP) of Virginia, designated CP-3 (Norfolk) and CP-10 (Dreweryville), and two in the West Central (WC) portion of the Virginia, designated WC-6 (Roanoke) and WC-7 (Salem). After running several gallons of water through the outlet pipe, fresh groundwater samples were collected at the four locations in 1-l Nalgene bottles. Some samples were fixed in formalin (1% final concentration) whereas unfixed samples were collected in sterile bottles for estimating numbers of metabolically active cells. Unfixed samples were stored in ice until they reached the laboratory. They were then incubated at 16° C prior to all experiments to restore ambient groundwater temperature, which ranges from 15 to 17° C. No more than 48 hours passed between collection and processing of live samples. Fixed samples were stored at room temperature.

DAPI Method:

Fifteen-ml samples of formalin-fixed groundwater were concentrated on 45-mm Nucleopore filters (0.2 μm pore size) pre-stained with Irgalan black. All enumeration experiments were run in triplicate. One ml of DAPI solution (Sigma Chemical Co., St. Louis, MO) (1 mg/100 ml) was added to each membrane filter and after incubation for 5 minutes the DAPI was drawn off by vacuum filtration. The membranes were rinsed with 2 ml of filter-sterilized distilled water containing formalin. The filters were

air-dried, mounted on glass microscope slides between two drops of immersion oil, and covered with No. 1 cover slips. Ten fields were counted per filter on an Aus Jena epifluoresence microscope under UV light at 350 nm.

INT-DAPI-FTF Method:

Fifteen-ml aliquots of unfixed groundwater were incubated with 1.5 ml 0.2% INT (aqueous) in sterile 250-ml screw cap bottles for 10 minutes at 16° C and then fixed with 0.15 ml formalin. The samples were then held overnight to allow the formazan deposits to coalesce within the cells. Samples were then stained with DAPI as described above. After the distilled water rinse, the membrane filters were placed cell-side down on microscope slides and wetted with one drop of the formalin-containing distilled water. The slides were placed on a block of dry ice for 10 minutes after which frost was gently removed with a Kimwipe and the filter was peeled off of the slide with forceps. A drop of glycerol-gelatin (Sigma Chemical Co., St. Louis, MO) was spread on one surface of a No. 1 cover slip which was then placed gelatin-side down on the slide over the cells. The cells were observed and counted on an Aus Jena epifluorescence microscope at 350 nm for DAPI stained cells. Brightfield microscopy was used for counting INT-formazan positive cells.

CFU Determination:

One- and 10-ml samples of unfixed groundwater were concentrated on Millipore membrane filters (0.45-μm pore size) and applied to plates of "4M" medium (0.2 g tryptic soy, 0.02 g yeast extract, 10 ml Hutner's Basal Salts (6), 15 g agar, 990 ml distilled water) in triplicate at each dilution. The 1-ml samples were mixed with approximately 5 ml of sterile distilled water prior to filtration for even distribution of cells on the filter. Plates were incubated 5 days at 16° C. Membrane filters with colony counts of 30-300 were counted and CFU/100 ml calculated.

Characterization:

Twenty isolates from each well were randomly selected and cultured on nutrient agar. After purification, the isolates were transferred to nutrient broth. Observations of live cells were performed under phase contrast microscopy with Nomarski optics to determine cell shape and motility. Gram stains were done routinely.

Results

Counts for WC-6 and CP-3 obtained by the DAPI method were twice as high as those for the other two wells (Table 1). These numbers agree statistically with those of a 1985 survey of the same wells using the same methods (unpublished data), with the exception of well CP-3 which had

three times the number of cells as it did in 1985. The total cell counts obtained by the INT-DAPI-FTF method agree statistically with the DAPI counts ($p = 0.0278$) (Table 1). This reinforces the accuracy of the INT-DAPI-FTF method. In the Coastal Plain (CP) wells, 20.57% (mean value) of the cells were INT positive, whereas in the West Central (WC) wells 17.58% (mean value) were positive. These means were significantly different ($p = 0.0065$).

The numbers of viable microorganisms, expressed as CFU, were extremely low in Virginia groundwaters, constituting less than 1% of the total cells counted by the DAPI epifluorescence method (Table 2). Although this gave a poor representation of all the bacteria present, the isolates were characterized based on cell shape, Gram reaction, and motility. Of the isolates cultured, 54.2% of the CP isolates were Gram-negative motile rods, while 87.5% of the WC isolates had these characteristics. This tendency toward a higher proportion of Gram-negative motile rods in the WC groundwaters is consistent with results obtained in 1985 (unpublished data).

Discussion

Enumeration of aquatic heterotrophic bacterial communities has been a historic problem (24). Most of these organisms cannot be accounted for accurately by plate count methods, so it was not until the advent of direct microscopic counting that reliable numbers could be determined.

Methods to differentiate live and dead bacterial cells began with Metchnikoff in 1887 (22) and have since been continuously improved and refined. Acridine orange is the most commonly used epifluorescent stain for differentiation of live and dead cells. Unfortunately, AO has several drawbacks, especially fluorescence of particulate debris. DAPI is a more specific fluorochrome because it complexes only with double-stranded DNA (20) and not with RNA or particulate matter. The double-stranded DNA is indicative of an intact, possibly functional genome. In addition, the DAPI staining technique is much quicker and less complicated than AO staining.

The given results with DAPI staining, however, agree well with those with AO staining for groundwater which were 6.82×10^4 cells/ml (4). Counts for groundwater communities from sediment samples are somewhat higher due to the addition of soil microorganisms (1,9,28). These three studies agree in the range of $1\text{-}10 \times 10^6$ cells/g dry sediment.

Although total DAPI counts indicate the number of potentially viable cells present, this does not indicate what proportion of these cells are metabolically active. Stevenson (25) provides an excellent argument for the dormancy of the majority of bacteria in aquatic systems. In 1978, Zimmermann (35) developed a method for the simultaneous count of total and respiring cells in a sample based on the reduction of INT. The theory behind this process is very sound, but one problem flaws this method. The red formazan deposit which develops as an indicator of a respiring cell is soluble in the immersion oil in which the cells are mounted for

microscopic observation. Consequently, the INT method has not been successfully used in groundwater (4). Attempts have been made to improve on this method (3,26) but they have tended to produce more problems than they solve. In order to make use of a combination of DAPI epifluorescent total counts and INT respiring counts, the filter-transfer-freeze (FTF) method (11) was used for the successful transfer of stained cells to the slide for observation. The modified method is more time-saving than the improvement reported by Tabor and Neihof (26) and is more reproducible. Controls revealed that the FTF technique resulted in nearly 100% transfer of the stained cells.

Values of 17-20% of total bacteria as INT positive are in the same ranges reported by other INT incorporation techniques for other aquatic ecosystems. For example, 6-12% of total bacteria was reported viable for the coast of the Baltic Sea and 5-36% of total cells in surface freshwater environments was viable (35). In other freshwater lakes and ponds 7.7 to 13.6% of the total bacteria were INT positive (17). These bacteria, however, were subject to the dissolution of some of the formazan deposits by the immersion oil and therefore these estimates are somewhat low.

The importance of direct enumeration techniques is emphasized by the failure of plate counting and other culturing techniques to account for a majority of the live cells present in a groundwater sample. Because the natural medium is so dilute, it is difficult to create an artificial medium capable of supporting the growth of all or a variety of the bacteria. Various media have been used to enumerate groundwater bacteria.

Spread-plating on enriched media (e.g., brain-heart infusion agar, casein-peptone-starch agar, and nutrient agar) has resulted in counts of 10 to 837 CFU/ml which at best represents 1% or less of the corresponding epifluorescent total counts (4). Other enumerations on dilute media averaged 118 to 310 CFU/ml (24) which agrees reasonably well with the present results of 3.5 to 185.3 CFU/100 ml. All of the reported results for groundwater samples counted from dilute media approximating the natural environment represented 1% or less of the direct counts and far less than that proportion indicated to be viable by INT reduction. These results illustrate the need for improved isolation techniques for aquatic microbiology. However, the implications are that improvements may still fall short of the desired goals, especially if approximately 80% of the microorganisms are in a dormant state, obligate oligotrophs, unable to grow on surfaces, or for some other reason defy colony--or large biomass--production. Herein lies the crux of future research.

Several studies including this one, confirm that Gram-negative organisms predominate in free flowing groundwater communities. My results indicate also that the majority of the Gram-negative bacteria present are motile, which may be significant. Through their motility, they may be capable of moving short distances to areas (interfaces) where substrates are more abundant. Moreover, Gram-negative bacteria contain various degradative enzymes in their periplasmic space, and this may contribute to the ability of the cells to use complex and varied nutrients. The future of

groundwater restoration after organic pollution may depend on these indigenous microorganisms.

Table 1. Estimates of the total populations of four Virginia groundwaters

Well No.	Population/ml (x 10^4)*	
	DAPI Method	INT-DAPI-FTF Method
WC-6	12.1 ± 3.3	11.6 ± 3.3
WC-7	4.95 ± 1.03	5.41 ± 1.54
CP-3	6.85 ± 1.6	5.21 ± 0.91
CP-10	4.0 ± 0.80	7.14 ± 0.21

*Mean ± standard deviation

Table 2. Estimates of viable populations in four Virginia Groundwaters

Well No.	CFU/100 ml*	CFU as % total viable pop. as estimated by DAPI staining	% of total viable pop. exhibiting dehydrogenase activity
WC-6	185.3 ± 37.6	0.085	16.4
WC-7	17.0 ± 1.3	0.12	18.75
CP-3	21.2 ± 0.4	0.01	20.8
CP-10	3.5 ± 0.2	0.012	20.33

*Mean ± standard deviation

CHAPTER THREE--SUBSTRATE UPTAKE BY HETEROTROPHIC GROUNDWATER BACTERIA

Introduction

The rates of substrate uptake and utilization are of primary importance to the cycling of nutrients in groundwater ecosystems. Numerous studies of this type have been done in other aquatic systems, including oceanic waters. The employment of ^{14}C-labeled organic compounds for uptake studies in freshwaters became widely known in 1966 with the work of Wright and Hobbie (34). They were the first to report widely the use of Michaelis-Menten enyzme kinetic calculations on the data resulting from addition of labeled organic compounds to aquatic samples; this enabled the calculation of maximum uptake velocity. They also stressed the importance of using low, near natural substrate concentrations. These researchers also improved the technique so that knowledge of the exact natural substrate concentration was not necessary to estimate the half-saturation concentration. Their method allowed calculation of a constant ($K_t + S_n$) equal to the sum of the natural substrate concentration (S_n) and the half-saturation concentration (K_t). This enabled researchers to study heterotrophic uptake of various substrates in a number of different ecosystems (2,10,13,30,34). Ladd et al. (16) studied the uptake of L-glutamic acid, L-phenylalanine, and glycolic acid in groundwaters of Alberta, Canada. In addition, they studied the degradation of radiolabeled lignocellulose. Ventullo and Larson (27) studied uptake by

using the turnover-time tracer method to examine the uptake of an L-amino acid mixture, D-glucose, L-arginine, and L-glutamate in samples from wells in Ohio and Ontario. The Canadian wells were subject to septic tank contamination whereas the US wells were unpolluted. They also studied the biodegradation of synthetic compounds, namely, 2,4-dichlorophenoxyacetate (2,4-D), sodium nitrilotriacetate, and benzoate.

This study was undertaken to measure the heterotrophic uptake of low molecular weight organic substrates common to intermediary metabolism in groundwater. Substrate concentration-dependent uptake of radiolabeled substrates was measured in four Virginia groundwaters. Substrate affinity, maximum uptake velocity (V_{max}), and half-saturation concentration plus natural substrate concentration constants ($K_t + S_n$) were examined to elaborate differences between microflora in different wells and in different geological regions. Four radiolabeled substrates were used in these studies.

Materials and Methods

After several gallons of water had passed through the outlet pipe, fresh groundwater samples were taken in sterile 1-l Nalgene bottles. Samples were promptly placed on ice and returned to the laboratory for immediate processing.

Samples of groundwater were equilibrated to their ambient temperature of 16° C and then transferred aseptically to sterile 100-ml serum bottles with butyl rubber stoppers. The total volume of the water plus radiolabeled substrate was 50 ml. Experiments were performed at least twice and in triplicate each time. Substrate was added to each sample, then it was shaken, incubated one minute, and fixed with 1 ml formalin. Two types of controls were used. An endogenous control without radioisotope but with unlabeled substrate was incubated and fixed identically to the radioisotope-containing samples. A second was a "zero-uptake" control, in which samples were formalin-fixed at least four hours prior to radiolabeled substrate addition. Respiration was measured in one series of experiments by absorption of ^{14}C carbon dioxide by published methods (19). In brief, the butyl rubber stoppers were fitted with center wells containing paper wicks. Phenethylamine was added to the center wells upon the acidification of the water samples. Samples were shaken two hours to enhance gas release. Each water sample was filtered onto a Nucleopore filter (0.2 μm pore size). The bottle was rinsed once and the filter twice with tap water to remove any unincorporated radioactivity. Filters and center wells were placed individually into 10 ml of Ecoscint (National Diagnostics, Manville, NJ). Samples were allowed to autofluoresce for 24 hours and then counted by liquid scintillation. Fixed control and endogenous control values were subtracted and counts were corrected by the channels ratio method. Calculations of V_{max} and (K_t + S_n) were made from Hanes plots (12). The concentrations of added substrate were plotted on the x-axis and turnover time on the y-axis.

Turnover time was calculated from the quantity ($C\mu t$)/c, where C = the counts per minute (CPM) of 1 μCi in the system (a correction factor), μ = μCi of activity added, t = incubation time, and c = the CPM taken up. The slope and y-intercept were calculated by linear regression analysis, for which only the linear portions of the Hanes plots were used. The slope equals the inverse of V_{max} and the y-intercept equals (K_t + S_n)/ V_{max}, which allows the calculation of the (K_t + S_n) value.

Results

Maximum uptake velocities (V_{max}) and natural substrate plus half-saturation concentration constants (K_t + S_n) were calculated from Hanes plots (Tables 3 and 4), based on the net uptake of the substrate. Values for kinetic parameters calculated for L-aspartate uptake in wells WC-6 and WC-7, are based on limited data (Figure 1). However, in the CP wells, L-aspartate uptake was more regular, with V_{max} values of 11.75 and 12.99 nmol/l/hr (Figure 2). Raw data for D-glucose, D-fructose, and succinate uptake are depicted in Figures 3,4, and 5, respectively. Kinetic parameters calculated for glucose uptake in well CP-10 are not statistically valid due to the erratic nature of uptake in that system. Trends were toward greater turnover time at higher substrate concentrations with the exception of L-aspartate uptake in the WC wells. In the WC wells, L-aspartate uptake peaked at a low concentration, whereas uptake of the other substrates in all wells peaked at much higher concentrations. Linearity coefficients for the closeness of the fit of the data to the

regression line and the significance levels are also provided (Tables 3 and 4).

Two sets of statistical analyses were performed with these data. The first compared the mean ($K_t + S_n$) values and mean V_{max} values for WC wells with those of CP wells for the four substrates. The mean V_{max} was significantly different in the two regions for glucose ($p = 0.0258$) and L-aspartate ($p = 0.003$). Only one mean ($K_t + S_n$) value was significanly different between the two regions, that of succinate ($p = 0.0470$). The second set of statistical analyses were multiple comparisons of mean V_{max} and mean ($K_t + S_n$) values between the four substrates tested. The mean V_{max} of L-aspartate uptake varied significantly from that of all three other substrates: succinate ($p = 0.007$), D-glucose ($p = 0.005$) and D-fructose ($p = 0.012$). Three ($K_t + S_n$) pairs showed a significant difference, succinate and D-glucose ($p = 0.085$), succinate and D-fructose ($p = 0.013$), and D-fructose and L-aspartate ($p = 0.024$).

Respiration was also measured but was not included in uptake calculations. The raw data for respiration are given in Appendix B.

Discussion

The free-flowing groundwater environment is one of relatively homogeneous and constant nutrient concentration. The nutrients are kept in even distribution by currents and diffusion (15). By performing kinetic investigations on microorganisms one can establish relationships between

Table 3. Uptake of Succinate and L-Aspartate in 4 Virginia Groundwaters

Well No.	Sub-strate	V_{max} (nmol/l/hr)	$(K_t + S_n)$ (nmol/l)	Correlation Coefficient	Sig. Level for line
WC-6	Succ	2.36	186.78	0.9669	0.0030
WC-7	Succ	2.93	251.13	0.9288	0.0118
CP-3	Succ	2.29	120.40	0.9133	0.0429
CP-10	Succ	3.10	123.18	0.7670	0.0717*
CP-3	Asp	11.75	116.30	0.9678	0.0029
CP-10	Asp	4.97	343.50	0.9772	0.0499
WC-6	Asp	1666.67	78.33	1.0000	0.0001
WC-7	Asp	2000.00	78.00	1.0000	0.0001

*Significant at the p = 0.10 level but not at p = 0.05

Table 4. Uptake of D-Fructose and D-Glucose in 4 Virginia Groundwaters

Well No.	Sub-strate	V_{max} (nmol/l/hr)	$(K_t + S_n)$ (nmol/l)	Correlation Coefficient	Sig. Level for Line
WC-6	Fruc	0.83	39.36	0.9628	0.0036
WC-7	Fruc	4.88	16.86	0.9943	0.0344
CP-3	Fruc	2.22	51.94	0.9191	0.0399
CP-10	Fruc	1.06	6.20	0.9541	0.0215
WC-6	Glc	1.21	79.09	0.9076	0.0459
WC-7	Glc	0.75	72.94	0.9696	0.0026
CP-3	Glc	1.91	94.12	0.9754	0.0105
CP-10	Glc	2.10	65.15	0.8762	0.1628*

*Not significant at the p = 0.05 or p = 0.10 level

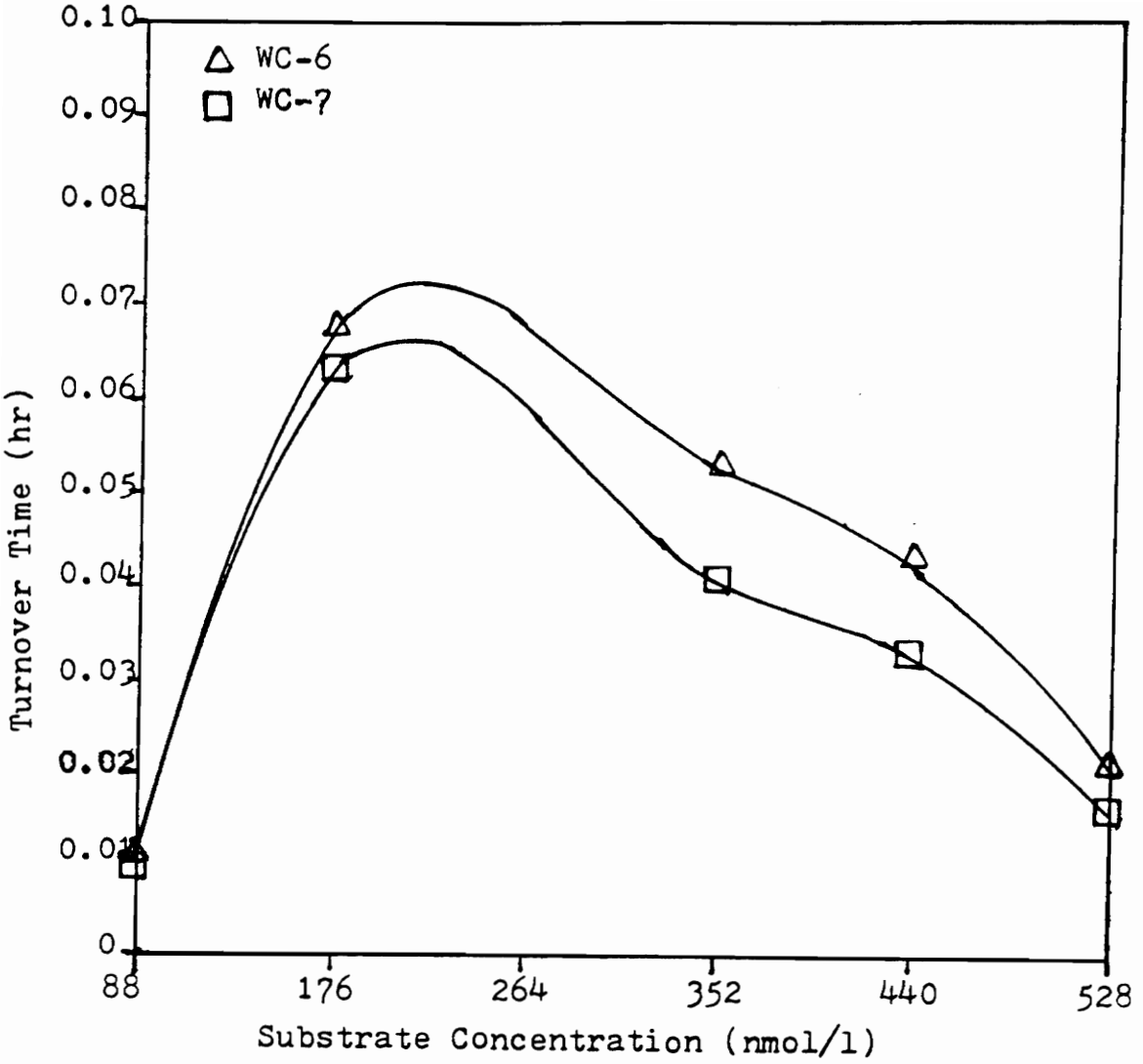

Figure 1. L-Aspartate Uptake in West Central Virginia Groundwaters

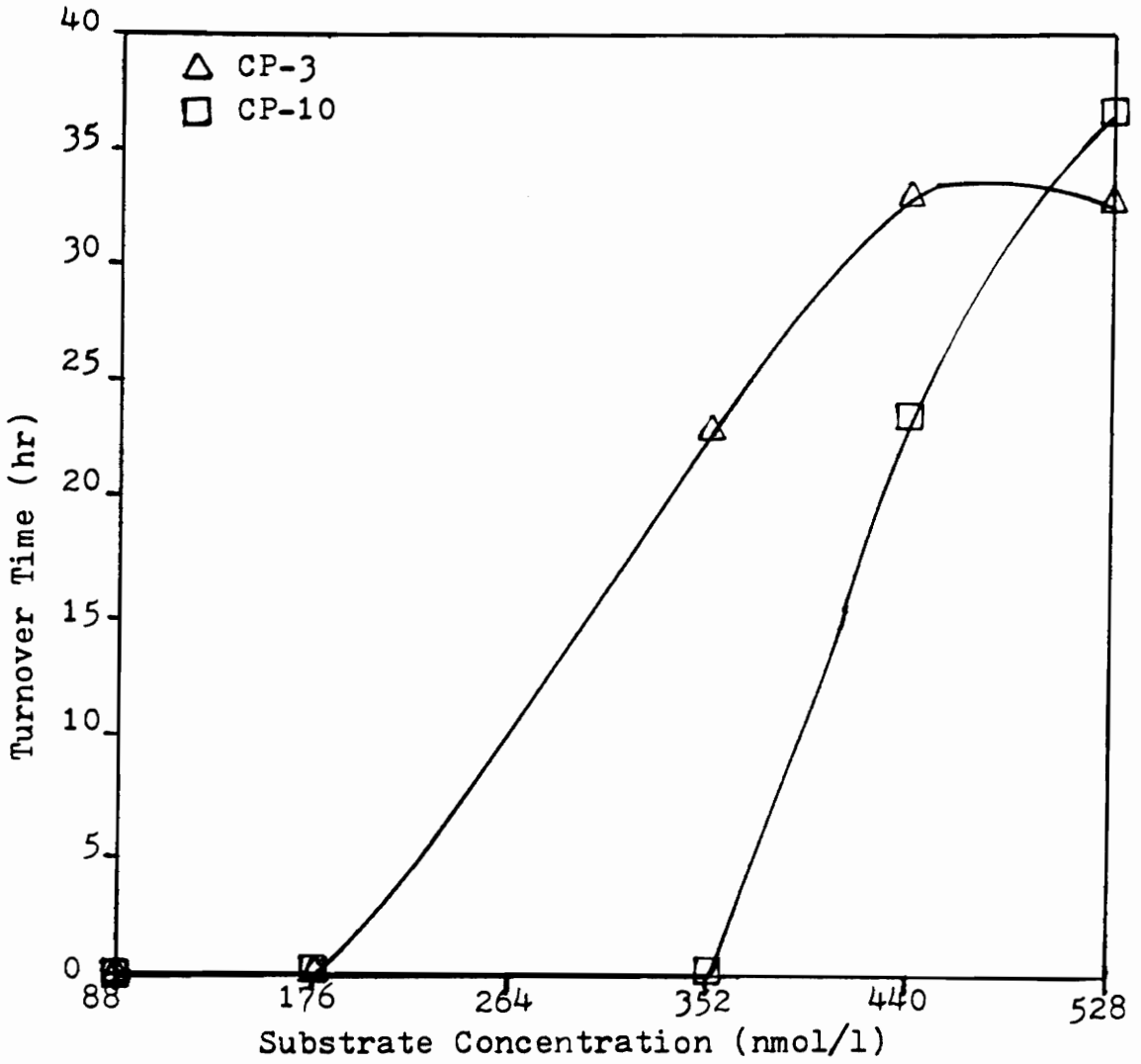

Figure 2. L-Aspartate Uptake in Coastal Plain Virginia Groundwaters

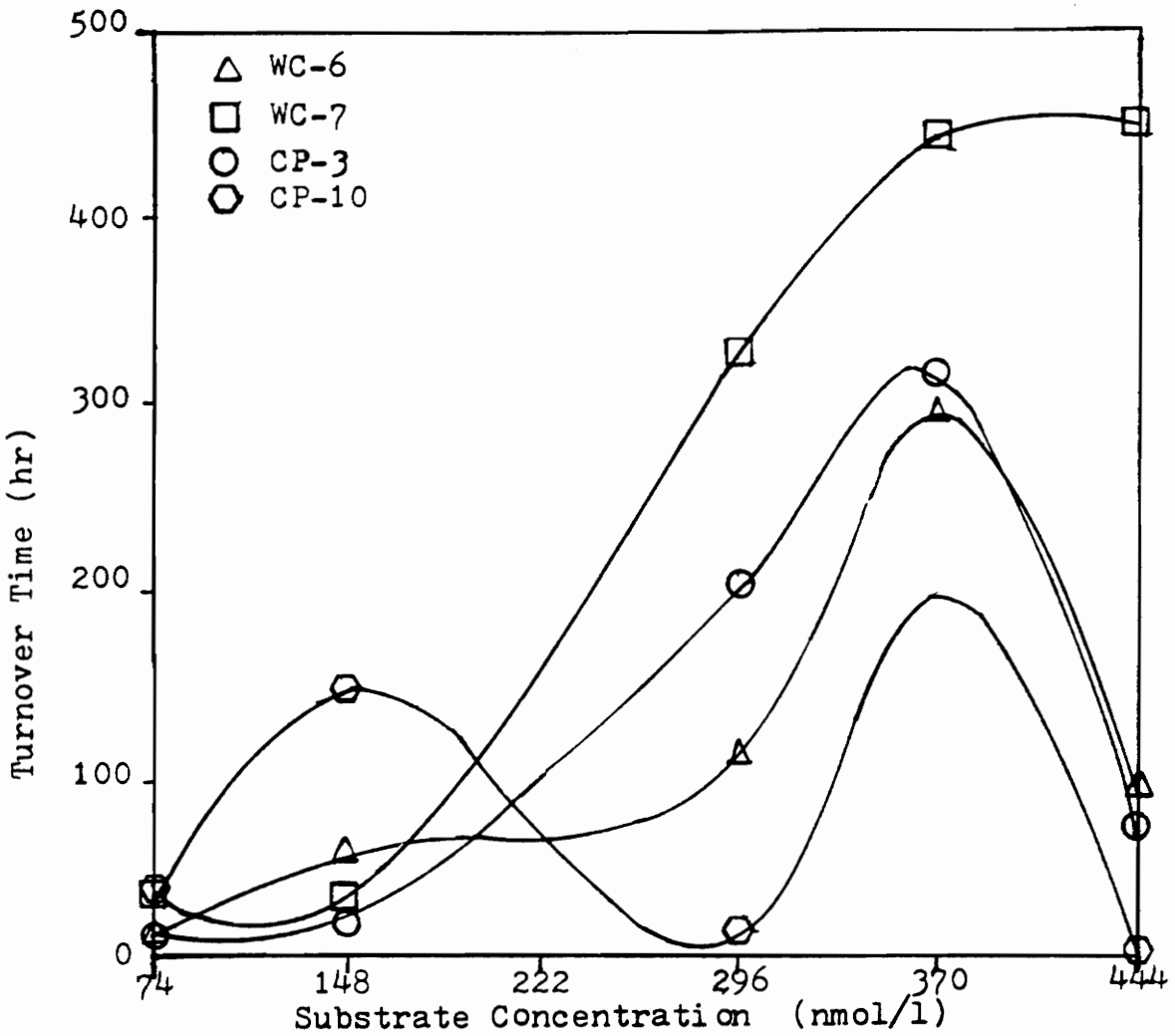

Figure 3. D-Glucose Uptake in Four Virginia Groundwaters

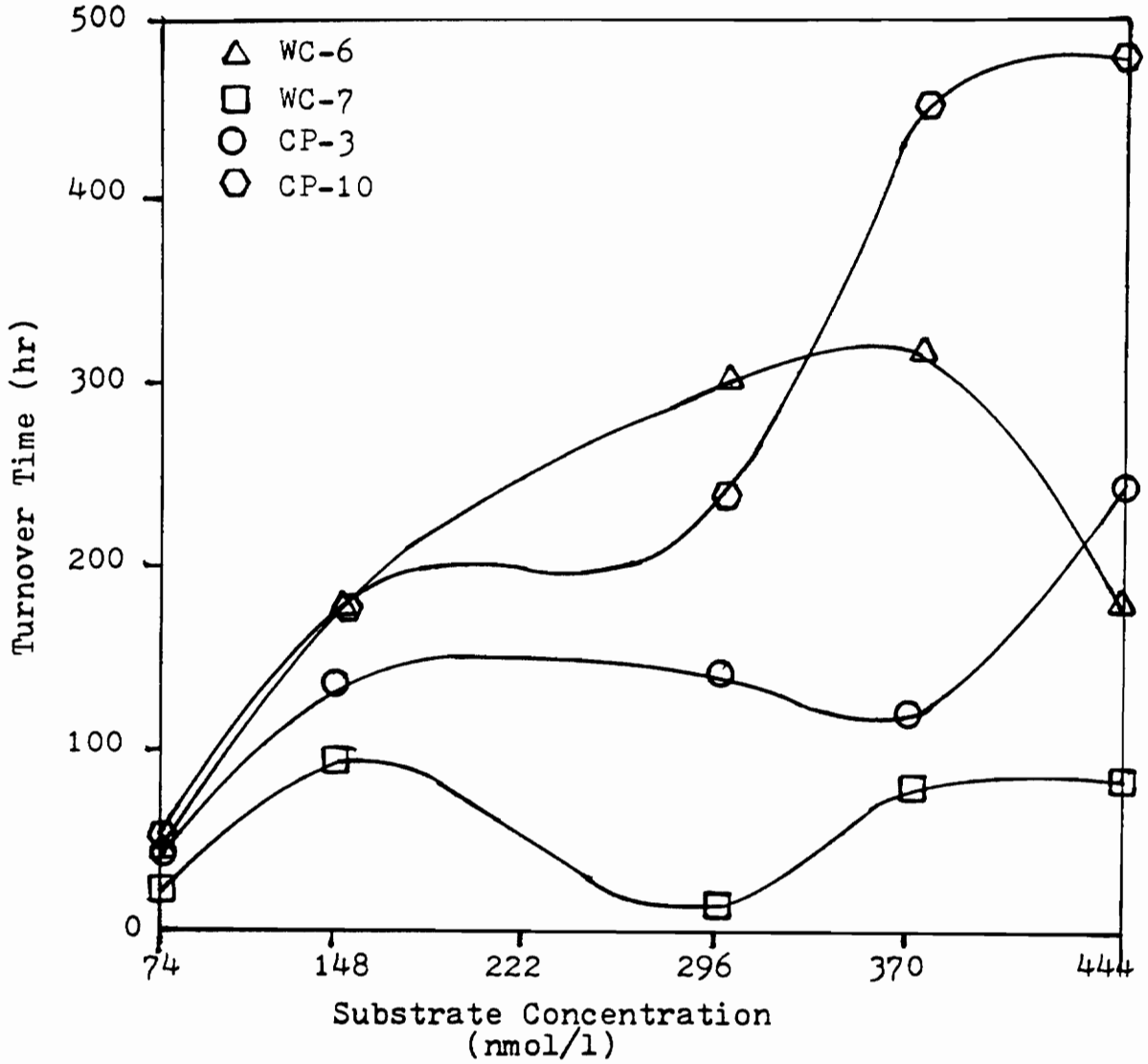

Figure 4. D-Fructose Uptake in Four Virginia Groundwaters

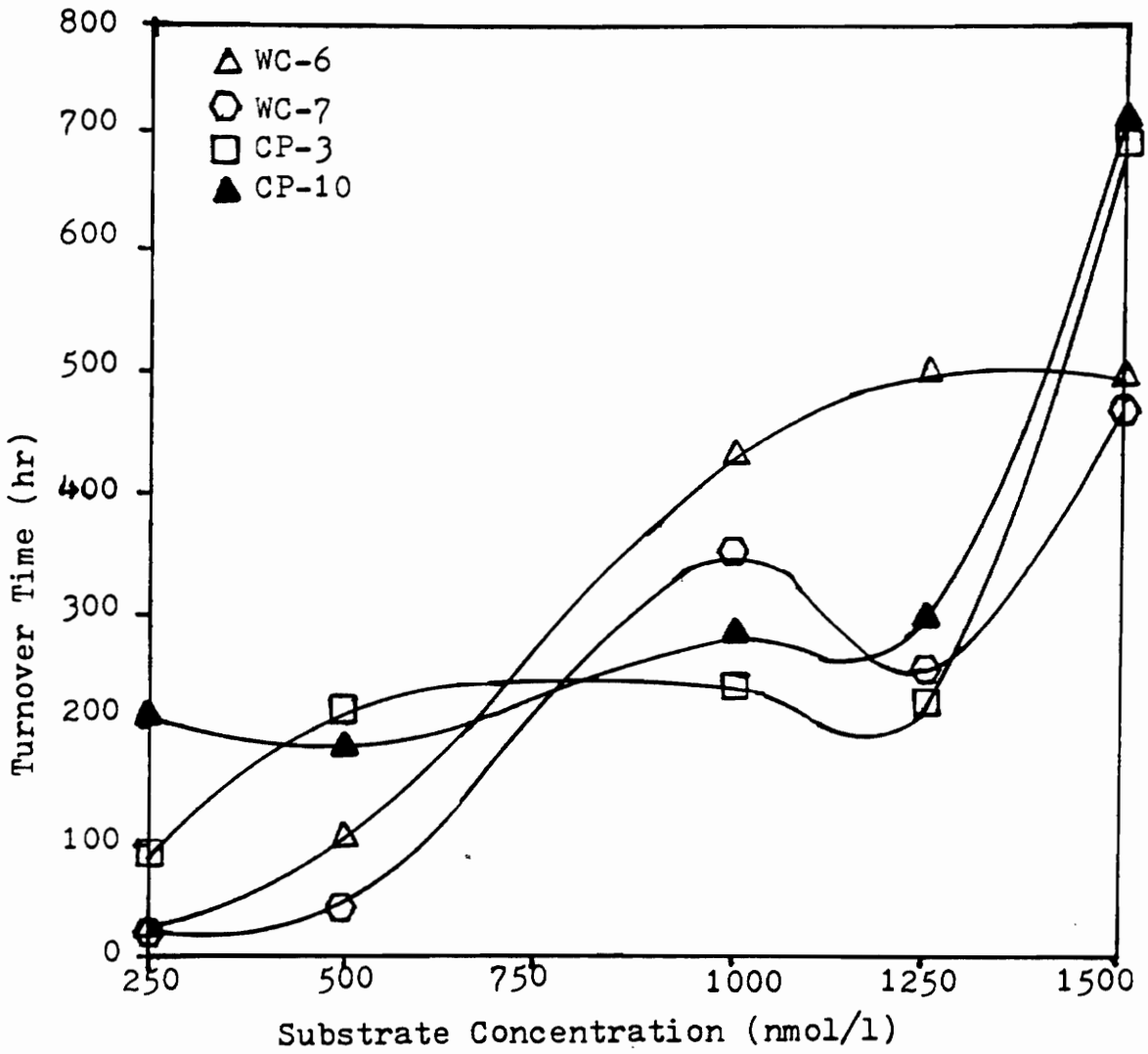

Figure 5. Succinate Uptake in Four Virginia Groundwaters

growth of bacteria and environmental factors; this is especially useful for determining the nature and amount of nutrients present (15). Because nutrient concentrations in groundwaters are very low, in the oligotrophic range of 1 to 15 mg organic C/l, the ability of certain bacteria to scavenge substrate when it is available only in low concentrations is a selective advantage (22). Kinetic studies of community systems are based on one very essential assumption. One must assume that all bacterial responses to the addition of substrate are basically the same; however, this does not occur (32).

The inhibition of L-aspartate uptake at the higher nutrient concentrations in West Central wells could be due to the relatively high concentration of L-aspartate which was added. These concentrations may be several orders of magnitude higher that the ambient L-asparate concentration. It is known that high nutrient concentrations in agar media can be inhibitory to indigenous oligotrophic microoganisms; a similar inhibition may be occurring here. The turnover time for L-aspartate in WC wells was very fast, i.e., had a low value, because so little of the substrate was taken up. The D-glucose uptake anomaly in CP-10 is unlike that of L-aspartate uptake in the WC wells. There are several possible reasons why the data plots were not linear, including the number of suitable transport systems available (5), and the heterogeneity of the indigenous community or the portion thereof which was active in each water sample.

There is only one reported set of data for heterotrophic uptake to which these results can be compared. Ventullo and Ladd (27) found that the mean for turnover rate (inverse of turnover time) was 0.0051/hr for glutamate, 0.004/hr for arginine, 0.0026/hr for an amino acid mixture, and 0.0049/hr for glucose. When the inverse of turnover time is calculated for Virginia groundwater results, the values fall in the same range. These results are 0.0015/hr for L-aspartate, 0.0004/hr for succinate, 0.0005/hr for D-fructose, and 0.0227/hr for D-glucose. Since natural substrate concentrations are not known for either set of wells it is impossible to say if any differences are a reflection of that parameter; however, that would be the most reasonable explanation. The total and viable cell counts are very similar. Total cell numbers are 4-12.1 x 10^4/ml in Virginia and 3.6-14.0 (except one well at 121) x 10^4/ml in the other sample sites (27). Also, by the INT-DAPI-FTF method, 0.8-2.0 x 10^4 viable cells are present per ml of Virginia groundwater whereas 0.2-11 x 10^4 viable cells/ml are present in the Ohio and Ontario wells based on MPN measurements (27).

The turnover rates are higher in WC wells than in CP wells for D-fructose, D-glucose and L-aspartate. Only with succinate was there quicker turnover in CP wells than in WC wells. Higher turnover rates in WC wells indicate a higher substrate affinity in the indigenous microflora. There are two possible explanations for this phenomenon. It may be that the microorganisms have adapted to their low-nutrient, more oligotrophic habitat by increasing affinity for substrate. Competition for the small amount of usable substrate available may have led to this selection. A

second possibility is that the greater population of Gram-negative motile rods makes the WC community physiologically superior to their CP counterpart, due to the greater number of cells that are motile and that harbor degradative enzymes in their periplasmic spaces.

Although these experiments have been limited in the number of wells and number of substrates studied, the results form a basis for some discussion. Complex high molecular weight pollutants would be more likely to be metabolized if they could be reduced to four-carbon compounds, especially those related to tricarboxylic acid cycle intermediates. Six-carbon intermediates do not appear to be as rapidly assimilated. Further study of amino acids rather than polysaccharides may prove more enlightening. For example, this might reveal that the TCA cycle may be used more for producing anabolic precursors than for catabolism. The most important question is whether these cells or others in their community could be capable of the primary breakdown of complex compounds to lower molecular weight substrates. The uptake data provided here could be combined with the knowledge of the degradative pathways for synthetic compounds; this may provide some insight into the amount of time that would be required for the restoration of polluted groundwaters using biologically oriented remediation techniques. At the very least, these experiments can serve as a starting point for the study of heterotrophic uptake of other substrates in groundwater systems.

CONCLUSIONS/SUMMARY

These results serve to reaffirm what is already known about bacterial groundwater communities and to further that knowledge. The total cells present in Virginia groundwaters correlate well with other groundwaters (4) as well as with oligotrophic marine environments where direct counts are 10^4 to 10^6 cells/ml (23). These numbers are 10 to 1000 times lower than those for more nutrient rich aquatic ecosystems such as estuaries and some eutrophic freshwater lakes and ponds (23).

The new method developed for enumeration of viable, i.e., INT positive cells is scientifically valid because the resulting counts agree well with those calculated from other methods (4,17). Moreover this method has several advantages. Unlike the other INT incorporation methods, the INT-DAPI-FTF method is rapid and fewer steps are involved with fewer opportunities for error. The other INT methods involved careful control of humidity or pH and careful timing, which are not required with this method (18,26). The use of the fluorochrome DAPI for both total counts and viable counting techniques has several advantages over the use of acridine orange. DAPI is more specific, no stepwise rinses are required and the total staining time is reduced (20). Thus, DAPI counts are more reliable and easier to obtain. The use of DAPI with the INT procedure adds one more advantage; the blue of DAPI fluorescence contrasts much better with the red-pink formazan deposits than does the red or green of acridine

orange. This enables better counts as well as easing eye strain. Neither DAPI, nor formazan dissolves in the glycerol-gelatin film, and the INT-DAPI-FTF method is preferable over the nalidixic acid method because the latter works only on Gram-negative bacteria. INT-DAPI-FTF is more reliable than slide culturing because attempts to use slide culturing in groundwater have had mixed results (4). Overall, the INT-DAPI-FTF is easy, gives valid results, and is not restricted to any one group of bacteria.

The results of heterotrophic uptake of low molecular weight organic substrates in groundwater are in accord with those of other oligotrophic waters, including other groundwaters studied (27). Based on the enumeration of the communities studied as well as the heterotrophic parameters that were calculated, groundwater communities share some common properties with marine (i.e., oligotrophic) ecosystems. In both systems, nutrient concentrations are very low, in the nanomolar range. Consequently, the organisms indigenous to these areas probably assume a dormancy when nutrient levels drop below those required for maintenance of vegetative cell life (25). This results in the high total counts but low viable counts found in both environments (15). The V_{max} values for several low molecular weight organic substrates fall into the same range for groundwaters as for marine environments, indicating that the heterotrophic communities in the two environments are metabolically similar (14,34). The high percentage of Gram-negative rods in each ecosystem may be an indication of their competitive ability due to their larger

surface area:volume ratio and more complex cell walls with their associated additional enzymatic and transport functions. The range of approximated K_m values of 1.16×10^{-7} to 6.1×10^{-9} mol/l indicates that these organisms have extremely high substrate affinities. Other K_m values of 10^{-7} to 10^{-8} mol/l are associated with high substrate affinity (33). Because a low K_m indicates a high affinity for substrate, the heterotrophic groundwater microorganisms are highly efficient at substrate uptake, which facilitates survival in a low-nutrient environment.

Several parameters indicate differences between WC and CP indigenous microflora. There are more total cells on average in the WC region but a lower percentage are metabolically active. Approximately 0.1% of WC bacteria as counted by DAPI staining formed colonies, whereas only 0.01% of CP cells could be cultured. Of the isolates, 87.5% of WC isolates and 54.2% of CP isolates were Gram-negative motile rods. The higher substrate affinities in the WC heterotrophic communities may be due, in part, to the higher proportion of Gram-negative motile bacteria present. Increased numbers of Gram-negative motile cells should result in the presence of more transport systems and degradative enzymes which would enhance the affinity of the bacteria for a substrate. Because they live in a less nutrient rich, more oligotrophic environment, WC heterotrophic microorganisms may have become better adapted to the low organic carbon level. They would respond rapidly to low levels of intermediary metabolism substances, but they might be shocked by the high concen-

trations or by refractile, poorly biodegradable pollutants. At this time, no one knows.

Now that these two environments are known to be similar in nutrient concentration and heterotrophic community composition and number, a logical next step is to apply other methods developed for marine systems to groundwaters. For example, the primary objective should be to develop an artificial medium analogous to artificial sea water, e.g., artificial groundwater, for more representative sampling of groundwater systems. Only with pure cultures can contributions to the community functions by individuals be determined (20). Also, once pure cultures of indigenous groundwater microflora are available, one can determine what degradative capabilities they possess, e.g., degradation of pectin, hemicellulose and cellulose, hydrocarbons, and other recalcitrant compounds. Contributions of groundwater bacteria to the cycling of nitrogen, sulfur, and phosphorus could also be studied in pure cultures (20). I also suggest long-term incubations which may provide further information on the reactivation of dormant cells upon substrate addition. This would further our knowledge of what is occurring in groundwater and which microorganisms are involved. Eventually it is hoped that enough information such as the results given here will be compiled to facilitate the prediction of organismal response to contamination and to indicated how the process of groundwater repair can be accelerated.

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APPENDIX A. SELECT DATA ON THE WELLS STUDIED

CODE: WC-6
LOCATION: Moose Lodge
NAME/OWNER: Roanoke Lodge 284

FORMATION: Braillier
LITHOLOGY: Shale
USGS QUADRANGLE REFERENCE: Salem
SCREEN DEPTH (FT):

SOLIDS (mg/l): 346.136
HARDNESS, EDTA (mg/l as $CaCO_3$): 252.364
pH (LABORATORY): 7.25
ALKALINITY/ACIDITY (mg/l as $CaCO_3$): 185.136
COD (mg/l): 2.286

ELEMENTS/COMPOUNDS
CALCIUM: 64.045 mg/l
TOTAL ORGANIC CARBON: 4.591 mg/l
CHLORIDE: 2.000 gm/l
COPPER: 0.428 mg/l
IRON: 1.697 mg/l
LEAD: 0.019 mg/l
MAGNESIUM: 19.164 mg/l
MANGANESE: 0.336 mg/l
NICKEL: 0.022 mg/l
NITROGEN (TOTAL KJELDAHL): 0.259 mg/l
AMMONIA (AS N): 0.168 mg/l
NITRATE (AS N): 0.065 mg/l
NITRITE (AS N): 0.00
PHOSPHORUS: 0.014 mg/l
POTASSIUM: 0.772 mg/l
SODIUM: 22.486 mg/l
SULFATE: 86.171 mg/l
ZINC: 0.968 mg/l

CODE: WC-7
LOCATION: Mitchell Dist. Co.
NAME/OWNER: Mitchell Dist. Co.

FORMATION: Elbrook
LITHOLOGY: Limestone
USGS QUADRANGLE REFERENCE: Glenvar
SCREEN DEPTH (FT):

SOLIDS (mg/l): 104.158
HARDNESS, EDTA (mg/l as $CaCO_3$): 94.474
pH (LABORATORY): 7.28
ALKALINITY/ACIDITY (mg/l as $CaCO_3$): 86.105
COD (mg/l): 2.769

ELEMENTS/COMPOUNDS
CALCIUM: 20.000 mg/l
TOTAL ORGANIC CARBON: 2.053 mg/l
CHLORIDE: 3.105 mg/l
COPPER: 0.243 mg/l
IRON: 0.030 mg/l
LEAD: 0.014 mg/l
MAGNESIUM: 7.926 mg/l
MANGANESE: 0.007 mg/l
NICKEL: 0.007 mg/l
NITROGEN (TOTAL KJELDAHL): 0.079 mg/l
AMMONIA (AS N): 0.00
NITRATE (AS N): 0.642 mg/l
NITRITE (AS N): 0.00
PHOSPHORUS: 0.00
POTASSIUM: 1.017 mg/l
SODIUM: 6.010 mg/l
SULFATE: 2.058 mg/l
ZINC: 0.124 mg/l

CODE: CP-3
LOCATION: Norfolk
NAME/OWNER: Compeco Cleaners
815 E. 26th Street
FORMATION: Upper Potomac Aquifer, Late Cretaceous
LITHOLOGY: Sand
USGS QUADRANGLE REFERENCE: Norfolk South
SCREEN DEPTH (FT): 820-830

SOLIDS (mg/l) TOTAL: 1601
VOLATILE: 117
FIXED: 1484
SUSPENDED SOLIDS (mg/l) TOTAL: 5⁻
VOLATILE: 5⁻
FIXED: 5⁻
HARDNESS, EDTA (mg/l as $CaCO_3$): 33
CONDUCTIVITY (micro mhos/cm): 2950
pH (LABORATORY): 8.3
ALKALINITY/ACIDITY (mg/l as $CaCO_3$): 392

ELEMENTS/COMPOUNDS

CALCIUM: 4500 ug/l
TOTAL ORGANIC CARBON: 14 mg/l
CHLORIDE: 620 mg/l
FLUORIDE: 2.19 mg/l
IRON: 50 ug/l
MAGNESIUM: 2700 ug/l
MANGANESE: 10 ug/l
NITROGEN (TOTAL KJELDAHL): 2.0 mg/l
AMMONIA (AS N): 1.2 mg/l
NITRITE + NITRATE (AS N): 0.05⁻ mg/l
NITRITE (AS N): 0.01⁻ mg/l
PHOSPHORUS (TOTAL): 0.2 mg/l
PHOSPHORUS (ORTHO): 0.13 mg/l
POTASSIUM: 16000 ug/l
SODIUM: 330000 ug/l
SULFATE: 71.6 mg/l

CODE: CP-10
LOCATION: Drewryville
NAME/OWNER: Town of Drewryville
LITHOLOGY: Sand
FORMATION: Middle Potomac Aquifer, midCretaceous
USGS QUADRANGLE REFERENCE: Drewryville
SCREEN DEPTH (FT):

SOLIDS (mg/l): TOTAL: 465
VOLATILE: 76
FIXED: 389
SUSPENDED SOLIDS (mg/l): TOTAL:
VOLATILE:
FIXED:
HARDNESS, EDTA (mg/l as $CaCO_3$):133
CONDUCTIVITY (micromhos/cm): 243
pH (LABORATORY): 7.1
ALKALINITY/ACIDITY (mg/l as $CaCO_3$): 143

ELEMENTS/COMPOUNDS

CALCIUM:
TOTAL ORGANIC CARBON:
CHLORIDE: 53 mg/l
FLUORIDE: 0.122 mg/l
IRON: 370 ug/l
MAGNESIUM:
MANGANESE:
NITROGEN (TOTAL KJELDAHL):
AMMONIA (AS N):
NITRITE + NITRATE (AS N):
NITRITE (AS N):
PHOSPHORUS (TOTAL):
PHOSPHORUS (ORTHO):
POTASSIUM:
SODIUM: 18000 ug/l
SULFATE: 5.82 mg/l

APPENDIX B. COUNTS PER MINUTE (CPM) FOR RESPIRATION OF LABELED SUBSTRATES

	L-Aspartate WC-6	L-Aspartate WC-7
88 nM	26661.9 ± 8604	0.0 ± 0.0
176 nM	174294.5 ± 29851.6	44876.3 ± 12391.2
352 nM	541830.1 ± 99144.4	212350.8 ± 25066.0
440 nM	864856.9 ± 28339.2	309309.1 ± 5953.7
528 nM	670453.6 ± 0.0	448493.0 ± 2955.8

	L-Aspartate CP-3	L-Aspartate CP-10
88 nM	0.0 ± 0.0	0.0 ± 0.0
176 nM	25896.1 ± 0.0	48909.0 ± 1731.4
352 nM	0.0 ± 0.0	0.0 ± 0.0
440 nM	0.0 ± 0.0	0.0 ± 0.0
528 nM	0.0 ± 0.0	0.0 ± 0.0

	Succinate WC-6	Succinate WC-7
250 nM	274.3 ± 378.1	354.1 ± 196.1
500 nM	184.1 ± 52.1	1334.5 ± 430.5
1000 nM	1550.4 ± 595.6	928.5 ± 566.8
1250 nM	1180.3 ± 879.6	481.7 ± 427.4
1500 nM	483.2 ± 264.3	3457.8 ± 2955.7

	Succinate CP-3	Succinate CP-10
250 nM	0.0 ± 0.0	985.1 ± 979.6
500 nM	0.0 ± 0.0	2500.2 ± 2241.9
1000 nM	174.3 ± 358.1	2918.5 ± 1922.9
1250 nM	0.0 ± 0.0	4500.4 ± 2464.6
1500 nM	18.3 ± 55.1	1615.1 ± 908.8

	D-Fructose WC-6	D-Fructose WC-7
74 nM	0.0 ± 0.0	695.0 ± 915.7
148 nM	0.0 ± 0.0	441.0 ± 699.9
296 nM	0.0 ± 0.0	1213.7 ± 1479.3
370 nM	537.8 ± 184.7	2170.4 ± 1997.5
444 nM	1039.2 ± 149.6	1576.9 ± 739.9

	D-Fructose CP-3	D-Fructose CP-10
74 nM	762.1 ± 0.0	765.1 ± 654.4
148 nM	55.7 ± 0.0	0.0 ± 0.0
296 nM	1636.3 ± 51.2	0.0 ± 0.0
370 nM	181.2 ± 0.0	0.0 ± 0.0
444 nM	4137.5 ± 1404.3	429.8 ± 569.7

	D-Glucose WC-6	D-Glucose WC-7
74 nM	393.3 ± 182.6	766.6 ± 369.2
148 nM	0.0 ± 0.0	0.0 ± 0.0
296 nM	696.4 ± 0.0	0.0 ± 0.0
370 nM	4476.8 ± 3575.3	0.0 ± 0.0
444 nM	1181.6 ± 337.3	0.0 ± 0.0
	D-Glucose CP-3	**D-Glucose CP-10**
74 nM	2004.4 ± 2044.8	0.0 ± 0.0
148 nM	5783.8 ± 5806.2	0.0 ± 0.0
296 nM	1800.9 ± 986.2	0.0 ± 0.0
370 nM	1626.7 ± 662.3	1182.0 ± 1685.1
444 nM	3281.2 ± 1256.4	0.0 ± 0.0

Appendix B. Counts per Minute (CPM) for Respiration of Labeled Substrates

APPENDIX C. LIST OF ABBREVIATIONS

AO	Acridine Orange
AODC	Acridine Orange Direct Counts
CFU	Colony Forming Units
CP	Coastal Plain
DAPI	2'4-diamidinophenylindole
FTF	Filter-transfer-freeze
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride
Kt + Sn	Half-saturation concentration plus natural concentration
MPN	Most Probable Number
SAI	Specific Activity Index
2,4-D	2,4-dichlorophenoxy acetate
Vmax	Maximum Velocity
WC	West Central

VITA

of

Laura Kathryn King

May, 1988

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PERSONAL

- Born: Greensboro, North Carolina
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EDUCATION

- Virginia Tech, June, 1986 Bachelor of Science
- Major: Biology Option: Microbiology Minor: Chemistry
- 1984-1986 Academic Dean's List
- Sigmund Sternberger Foundation Scholarship Recipient 1982-1986

RESEARCH INTERESTS

Environmental and Applied Microbiology, specifically the response of indigenous heterotrophic groundwater bacteria to low organic substrate availability

PROFESSIONAL EXPERIENCE

- Phycology Teaching Assistant, 3/88-5/88 Virginia Tech
- General Microbiology Teaching Assistant, 9/87-3/88 Virginia Tech
- Research Assistant, 6/87-9/87 Virginia Tech
- General Biology/Principles of Biology T.A. 9/86-6/87 Virginia Tech
- Undergraduate Researcher/Coordinator 6/85-6/86 Virginia Tech

MEMBERSHIPS

- American Society for Microbiology
- Society for Industrial Microbiology

PUBLICATIONS

- King, Laura K. 1987. CATGEN: A Software Review. Journal of Computers in Science and Mathematics Teaching. 4:77-78.
- Jervis, C.K. and L.K. King. 1988. A preliminary survey of computer use by freshman level college students. VA J. Science In press.

- King, Laura K. and Bruce C. Parker. 1988. A simple, rapid method for enumerating total viable and metabolically active bacteria in groundwater. Appl. Environ. Microbiol. In press.

PRESENTATIONS

- Characterization and Nutrition of Oceanic Planktobacteria, Virginia Tech, Sept. 30, 1987.
- Response of Indigenous Heterotrophic Groundwater Bacteria to Low Organic Substrate Availability, Virginia Tech, April 19, 1988.
- Heterotrophic Uptake of Low Molecular Weight Organic Substrate by Groundwater Bacteria. ASM National Meeting, May 10, 1988.