ISOLATION, IDENTIFICATION, AND CHARACTERIZATION OF GROUND WATER BACTERIA

by

Linda Dale Allen Stetzenbach

A Thesis Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

1 9 8 4

STATEMENT BY AUTHOR

This thesis has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or part may be granted by the head of the major department or the Dean of the Graduate College when in his judgement the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: _____

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

_____
N.A. Sinclair
Professor of Microbiology

4/30/84
Date

To Klaus and our little angels, Erika and Kristen,
for their love and understanding

ACKNOWLEDGEMENTS

I wish to thank the faculty and staff of the Department of Microbiology and Immunology for their guidance and support during the course of this research. In particular I wish to acknowledge the encouragement of Dr. N.A. Sinclair and Dr. C.P. Gerba, and the assistance of Dr. L.M. Kelley with the statistical analysis of the data. My thanks also to Marylynn V. Yates for her help with the collection of the water samples and for her invaluable instruction on the writing of this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES--continued

LIST OF ILLUSTRATIONS

ABSTRACT

Ground water is an increasingly significant source of potable water and is one of the least studied ecosystems on earth. Assessment of man's impact on this resource requires knowledge of the microbial populations present. Over 500 bacteria were isolated from well water samples on a low nutrient medium (R2A). Gram-negative, rod shaped, non-motile bacteria predominated. Acinetobacter spp. comprised 54% of the total isolates. Direct counts using epifluorescent microscopy revealed that representative isolates grew significantly in filtered well water. Growth of Acinetobacter sp. was significantly enhanced in water enriched with low concentrations (100 ug carbon/liter or 1000 ug carbon/liter) of glucose, acetate, succinate, or pyruvate. The growth and survival of an Acinetobacter sp., an opportunistic pathogen, in water had not previously been described. Bacterial survival studies conducted on water samples from 19 wells throughout the Tucson basin revealed the presence of a significant population of bacteria capable of rapid growth and survival in unamended native well water.

INTRODUCTION

Ground water accounts for over 90% of the world's fresh water resources (Scalf et al.,1981), and is recognized as an important worldwide source of water for irrigation, industry and human consumption (Todd, 1980). Once thought to be a primary resource only in arid regions, the use of ground water has increased to include all climatic zones (Murray and Reeves, 1977). Trends of ground water withdrawal in the United States show an increasing proportion of the total water utilized is from ground water sources (Murray and Reeves, 1977 and Freeze and Cherry, 1979). Well water constitutes the sole water source in many regions (Todd, 1977) and approximately 1/2 of the U.S. population relies on ground water as the principle source of potable water (Zaporozec, 1979). With increasing demand for high quality potable water, projections indicate that by the year 2000, ground water usage would reach 33% of the total water used in the United States (Freeze and Cherry, 1979).

While assessment of water quality traditionally has included the monitoring of the chemical constitutents and physical properties associated with drinking waters, biological aspects have been limited to the detection of coliform bacteria as indicators of potential fecal pollution (American Public Health Association, 1980). Information

concerning the non-coliform bacteria present in well water is limited and for the most part restricted only to the enumeration of organisms referred to as heterotrophic plate count (HPC) bacteria. The identification and characterization of these microbial populations present in ground water has largely been overlooked.

Early studies in microbial ecology indicated that populations of microorganisms decreased with increasing depth (Waksman, 1916). This assumption combined with the supposed protection of the deep subsurface confered by the soil mantle was believed to result in ground water of high bacteriological quality. The presence of bacteria in regions once thought to be devoid of life has kindled interest in the presence of microbes deep within the earth's crust and underlying aquifers. While some extreme instances do exist, the overall effects of temperature, pH, pressure, and chemical components within subsurface environments have been shown to be compatable with microbial life (McNabb and Dunlap, 1975). Micro environments are now believed to be actively inhabited by microorganisms virtually throughout the subsurface.

The chemical characterization of ground water indicates that nutrients available to microorganisms are in relatively low concentrations (Matthess, 1982). It is the concentration of dissolved, easily assimilated organic substances that serves as the most important factor in the

establishment of a microbial community in a region of limiting nutrients (Kuznetsov et al.,1979). Microorganisms that inhabit the subsurface, either as indigenous microbes or those introduced at well drilling, must therefore be capable of survival and growth at nutrient concentrations far less than those present at the surface. Such environments may require cells to develop specific morphological and/or physiological adaptations. Many aquatic microorganisms are periphytic with the development of bacterial filaments. These filaments are produced during times of severly limiting nutrients and provide the cell with a means of attachment to surfaces where the nutrient concentration is higher (Kuznetsov et al., 1979 and Poindexter,1981). The development of prostheca containing enzymes related to both transport and oxidation is stimilated by the lack of organic substances or phosphorous and results in increasing diffusion rates (Kuznetsov et al., 1979 and Larson and Pate, 1975). Mechanisms for survival under starvation conditions may also include a reduction in cell volume with an increase in the surface to volume ratio. Changes in size and shape (e.g. rod-sphere morphogenesis) have been observed in a marine vibrio grown in a low nutrient medium (Novitsky and Morita, 1976). Rapid reduction in cell size was observed during the first 2 days. Inital cells were 1 um x 4-5 um. After 2 weeks, 100% of viable cells passed through 1 um filtration. After 3 weeks, 100%

passed through Ø.6 um pore filters, with 5Ø% filterable through Ø.4 um filters. Efficient replication permitting cells to increase in number without increase in biomass combined with reduced respiration rates may allow the microbial community to conserve available carbon (Novitsky and Morita, 1978). Over 8Ø% reduction in endogenous respiration was observed for a psychrophilic marine vibrio during the first 2 weeks of starvation. The organism also demonstrated an increase in the number of nuclear bodies per cell. Utilization of reserve materials and cellular constitutents resulted in an observed 4Ø% decrease of cellular protein and allowed survival for extensive periods of sparse nutrient conditions (Kuznetsov et al.,1979 and Novitsky and Morita, 1978).

Cryptic growth (i.e. viable cells utilizing nutrient components of dead cells) has also been studied as a means for survival. Heat killed cells of Salmonella enteritidis, an organism normally isolated from environments rich in nutrients (ie. the intestine of man and animals) (Buchanan and Gibbons, 1974), has been theorized to support living cells at a rate of 6.7 heat killed cells / viable cell (Druilhet and Sobek, 1976). It is this large number of dead cells needed to support the doubling of a remaining cell that has led researchers studing environmental isolates to discount cryptic growth as a mode for survival in dilute environments (Novitsky and Morita, 1978).

Increased numbers of marine heterotrophic bacteria have been isolated using media low in nutrient content (Akagi, 1977, 1980) and media concentrations as low as 0.1 mg carbon/liter have resulted in growth of some marine heterotrophs (ZoBell and Grant, 1942). Bacteria capable of life in medium containing $\leq$15 mg carbon/liter are termed oligotrophs. It is their ability to survive and grow at nutrient concentrations less than 15 mg carbon/liter that insures the establishment of oligotrophic bacterial populations (Kuznetsov et al., 1979).

Once established, the oligotroph may serve several roles in nature. As the ultimate scavangers of materials which would otherwise be sequestered, autochthonous populations of oligotrophs may alter the fate of pollutants introduced into the subsurface (McNabb and Dunlap, 1975). Potential pathogenic microorganisms capable of life as an oligotroph when introduced into the subsurface may alter the potability of the water. Frank and opportunistic human pathogens have been isolated from rural drinking water wells. Staphylococcus aureus, Actinomycetes sp., Micrococcus luteus, Aeromonas hydrophilia, Acinetobacter calcoaceticus, Alcaligenes sp., Flavobacterium sp., Moraxella sp., and several members of the genus Pseudomonas (Lamaka et al., 1980) have been identified. Flavobacterium sp. and A. hydrophilia have been shown to grow in water with low

concentrations of available nutrients (van der Klooij et al., 1980,1981). Studies with Pseudomonas cepacia have shown that this organism was able to grow and survive in distilled water (Carson et al., 1973). Acinetobacter calcoaceticus, an organism that has been frequently isolated from soil and water, requires only simple organic compounds as the sole sources of carbon and energy (Hendiksen, 1973).

The opportunistic pathogens Flavobacterium spp., Aeromonas spp., Pseudomonas spp., and Acinetobacter spp. are not detected by current standard methods. Their introduction into the aquifer could lead to the establishment of resident populations capable of significant impact. Several of these bacteria have been shown to interfer with the detection of coliforms during standard water quality analysis (Geldreich et al., 1978, Hutchinson et al., 1943, and Herson and Victoreen, 1980). The study of indigenous microbial populations and the identity and characterization of non-coliform bacteria in well water therefore is essential in understanding the overall quality of untreated waters.

Investigators have used several approaches in the study of aquatic microorganisms. Maintainance of laboratory conditions approaching those of the in situ environment is essential. Henrici (1933) and Henrici and Johnson (1935) immersed glass slides in water for several days, resevoir

water serving as a natural nutrient medium. Studies involving growth rates and metabolism of periphytic bacteria using glass slides viewed with phase microscopy was adopted by Bott and Brock (1970). The use of membrane filtration for the concentration of microorganisms improved on the idea of direct count studies however there was some difficulty in distinguishing particulate matter from small bacteria. Enhanced resolution has been demonstrated with fluorescent staining and epifluorescent illumination (Daley and Hobbie, 1975). Bacteria treated with acridine orange (AO) when viewed with epifluorescence has resulted in counts of small (0.2-1.0 um) organisms which were not viewed when the filters were dried, cleared and viewed with transmitted light. The use of nuclepore filters (Nuclepore Corp.,Pleasanton, Ca.) and epifluorescent microscopy to further enhance the retained cells results in a reliable direct count method (Brock, 1978 and Hobbie et al., 1977). Epifluorescence has also been used to study bacterial growth rates. With this microscopic technique, the frequency of dividing can be determined by counting 300 cells and recording the percentage of cells with a clear intervening wall. A direct relationship can be drawn to the microbe's growth rate (Hagstrom, 1979). The growth rate can then be used to calculate productivity and assess metabolic activities of aquatic bacteria (Pedros-Alio and Brock, 1983).

Culturing of aquatic microorganisms presents a variety of problems since the organic content of the culture medium is critical to the isolation of a truly representative oligotrophic microbial population (Kuznestov et al., 1979).

Chemostats have been used for the study of oligotrophs with the exhaustion of limiting nutrients and the accumulation of metabolic products eliminated by the constant input of precise nutrient levels and the constant removal of cells and products (Jannasch, 1967a,b).

Investigators have proposed media formulations consisting of little more than trace salts, water, and an organic content of 0.05-0.3% in the study of oligotrophic microorganisms (Carson et al., 1973, Larson and Pate, 1975, and Poindexter, 1981). Akagi (1977) demonstrated the use of a glass-fiber Nuclepore filter with a low nutrient medium in lieu of agar solidified plate methods for the enumeration of oceanic isolates. Diluted soil-extract medium (DSEA) prepared from 100 grams of surface soil in 100 ml distilled water, autoclaved and solidified with agar has been used for culture of ground water bacteria (Wilson et al., 1983). Plate counts however were highly variable. Standard Methods Agar (SMA) (BBL, Cockeysville, Md.) has been used in the cultivation of the aerobic and faculative anaerobic bacteria present in waters (American Public Health Assoc., 1980). SMA, however, contains approximately 8.5 grams/liter of

utilizable nutrients for carbon and energy. This concentration is far in excess of the recomended levels for the isolation of oligotrophs (Kuznetsov et al., 1979). R2A (appendix) consists of a variety of carbon and energy sources at low concentrations (approximately 2.5grams/liter) and has been shown to be a superior isolation medium for fresh water bacteria (Reasoner and Geldrich, 1979).

Once isolated, the identification of the majority of heteroptrophic environmental microorganisms has been achieved using common biochemical tests. Computerized rapid identification methods (e.g. API) however, rely primarily on clinical isolates for the data base. Methods for the identification of Gram-negative rod shaped bacteria using a variety of individually prepared media (e.g. King chart) require an extensive investment in preparation, inoculation, and reading time. The Lassen (1975) three-tube method was developed for the rapid identification of Gram-negative rods with a minimum number of tests.

Isolation of oligotrophs from well water, and evidence of metabolism at low nutrient as indicated by growth studies have provided data to support the contention that these organisms can colonize in water (van der Kooij, 1980, 1981) and in the subsurface (Wilson et al., 1983).

The purpose of this study was to isolate and identify the heterotrophic bacterial populations present in Tucson well water and to monitor the growth and survival of

representative isolates in water. The impact the presence of these microorganisms may have on the subsurface environment and on the quality of drinking water cannot be ignored.

MATERIALS AND METHODS

Water Sources and Sampling Sites

Two continously pumping wells with similiar chemical and physical characteristics were selected as sampling sites for bacterial culture studies. Samples were collected over a 90 day period from April 1982-July 1982.

Nineteen continously pumping deep ground water wells throughout the Tucson basin were also selected for studies of bacterial survival in well water.

Sample Collection

Well water was flushed through the wells' sampling port for 2-3 minutes prior to collection in sterile 1 liter wide-mouthed polypropylene bottles. Samples were placed on ice and returned to the laboratory for processing within 1-2 hours of collection.

Chemical and Physical Characterization of Well Water

Water temperature was recorded at time of collection. Turbidity and pH were determined in the laboratory. Turbidity was measured using a HACH turbidimeter (Model 2100A). A Beckman Expandomatic SS-2 was used for pH determinations. Data for water chemistry and trace metals were provided by the University of Arizona Physical

Resources Department citing records of the Department of Health Sciences State Laboratory.

Enumeration and Classification of Isolates

Water samples (Ø.1 ml) were spread in triplicate onto R2A media. Cultures were incubated aerobically at 26 C for 7 days and colonies were enumerated with the aid of a stereoscope (35x). The number of colony forming units (cfu) were recorded and the plate most representative of the mean colony count was selected for further study. Colony sizes and pigmentation were recorded and the isolates were subcultured for pure culture isolation onto R2A. Subcultures were incubated for 5 days at 26 C. Pure cultures were first classified according to their Gram reaction and morphology. Gram-positive rods and Gram-positive cocci were not tested further.

An experiment using duplicate Ø.1 ml samples were inoculated in triplicate onto plates of R2A and Standard Methods Agar (SMA). The R2A plates were incubated at 26 C for 7 days. The SMA plates were incubated for 3-5 days at 35 C. Enumeration and identification of isolates proceeded as outlined above.

Biochemical Identification of Gram-negative Rod-shaped Bacteria

All isolates were characterized using the three tube rapid identification method of Lassen (1975) (appendix).

Kligler`s Iron Agar (KIA) was substituted for the combined lactose-glucose-H2S tube described as tube #1. KIA confirms the fermentation of glucose and/or lactose, allows for the detection of H2S production (Difco, Detroit, Michigan). Motility in Ø.25% agar and utilization of mannitol was detected using the mannitol-motility (MM) described as tube #2. Tube #3, urea-indole (UI) broth was included in the scheme for the detection of urease and/or the production of indole. Oxidase reaction was determined with filter paper saturated with N,N,N,N'-tetramethyl-p-phenylene diamine dihydrochloride (Alfa Products, Damers, MA). Isolates were streaked onto the filters with wooden applicator sticks. Purple to black discoloration within 1Ø seconds constituted a positive reaction (MacFaddin, 198Ø).

Growth Studies

Selected isolates of Acinetobacter sp., PO, and Moraxella sp. were cultured at 26 C in well water filtered through a Ø.2 um filter (Nuclepore) and serially transfered to freshly collected and filtered well water (FW) at 72 h intervals over a 3Ø day period. These cultures were then used as stock suspensions for the subsequent experiments.

One ml of stock suspension was inoculated into 125 ml Erlynmeyer flasks containing 1Ø ml FW. Cultures were incubated at 26 C on a wrist-arm shaker.

Experiments with FW amended with low concentrations of organic carbon were also performed. Carbon sources including glucose, pyruvate, acetate, and succinate were prepared separately in 10x concentrations, filtered through 0.2 um filters and stored at 6 C in acid-washed glass bottles. The carbon sources were added separately to FW in final concentrations of 100 ug carbon/liter and 1000 ug carbon/liter respectively.

All glassware used in growth studies was soaked 2-3h in concentrated H2SO4 with trace KMNO4, rinsed 3 times in distilled water and dried overnight in a hot air oven at 90 C to remove all traces of organic carbon. Glassware was then cooled and stoppered with sterile cotton and stored until needed.

Bacterial growth was determined by epifluorescent microscopy (Nikon, Optiphot). Samples were removed at intervals and bacteria were enumerated using the acridine orange direct count (AODC) technique of Hobbie et al. (1977). Acridine orange (AO) (0.1%) in citrate buffer was filtered (0.2 um filter, Nuclepore) and stored at 6 C in an acid washed bottle until needed. The bottle was wrapped with foil to exclude light. Polycarbonate 0.2 um pore size filters (Nuclepore) were prestained with 1% filtered nigrosin solution for 10-24h and rinsed with filtered distilled water just prior to use. A 2 ml sample of culture to be stained was mixed with 0.2 ml AO and after

approximately 2 minutes holding at room temperature the entire volume was filtered through a prestained filter. The filter was rinsed with 5 ml distilled water and immediately placed on a glass slide. One drop of 1% nigrosin mixed with and equal volume of glycerin was placed on the wet filter and a cover slip was applied. The number of bacteria per ml were estimated using the guidelines of Krane and Sutter (1962). A minimum of 200 fluorescent cells were counted per slide which yielded a 15% error at the 95% confidence limit.

Survival of Bacteria in Well Water

Water samples were collected as described above from 19 continously pumping wells. Twenty-five ml volumes were asceptically transferred to sterile polypropylene tubes (Falcon, Oxnard, California) and incubated at in situ well temperature. Bacteria were enumerated by AODC at predetermined intervals for 30 days following the procedure outlined above.

Heterotrophic plate counts (HPC) were determined by the spread plate technique using R2A medium. Cultures were incubated aerobically for 5 days at 26 C, and colonies were counted. Selected isolates were tested for oxidase activity.

RESULTS

Well Site Data

Well site data and water quality analysis of the 2 wells selected for bacterial culture studies are presented in Table 1. The wells are located on the campus of the University of Arizona, Tucson, Arizona. Water quality analysis for the 2 wells revealed similar characteristics and the wells are believed to draw from the same three aquifer layers (William Witchie, University of Arizona Division of Physical Resources,personal communication). The 19 wells selected for the bacterial survival are located throughout the Tucson basin. Well site data were obtained from records of the City of Tucosn Water Department (Table 2). Turbidity, pH, and HACH kit determinations of water quality for the 19 water samples is presented in Table 3.

Enumeration of Heterotrophic Bacteria from Well Water

Well #1 averaged 236 cfu/ml well water while Well #2 averaged 508 cfu/ml (Table 4). Eleven samples were collected during the study from Well #1 and 5 samples collected from Well #2 (Table 5 and 6).

Table 1. Well Site Data

	WELL #1	WELL #2
Depth (meters)	151	182
Water Level (meters)	60	60
Flow (gpm)	200	350
Well casing	steel, 20" diameter	steel, 18" diameter
Drilling Date	1937	1966
Water Temperature	26 C	26 C
pH	7.7-7.9	7.8-7.9
Turbidity (ntu)	0.10-0.25	0.16-0.31
Water Quality Analysis (mg/L)		
Calcium	46	36
Magnesium	4	2
Sodium	44	47
Iron	<0.1	<0.05
Copper	<0.01	<0.05
Manganese	<0.05	<0.05
Zinc	<0.05	<0.05
Alkalinity	92	106
Chloride	18	10
Nitrate	11.5	5
Sulfate	62.5	62.5
Fluorides	0.19	0.18
Residue	299	272
Hardness (CaCO3)	132	96
Barium	<0.5	<1
Arsenic	<0.02	<0.005
Silver	<0.005	<0.005
Chromium	<0.01	<0.01
Cadmium	<0.005	<0.005
Lead	<0.02	<0.02
Selenium	<0.005	<0.005
Mercury	<0.0005	<0.0005

Table 2. Well Site Data of 19 Tucson Wells*

Well	Date Drilled	Discharge (gpm)	Bowl Depth (ft)	Pumping Level (ft)	Casing Diameter (in)
B3	1974	212	260	235	16
B42	1977	650	350	275	16
B45	1977	707	280	204.9	16
B75	1964	1538	250	217.3	16
B86	1968	559	240	192.6	16
C48	1977	923	320	265.1	16
C49	1976	977	300	255.2	16
C56	1976	736	330	289.4	16
C76	--	553	200	135.5	12
C87	1956	403	300	263.8	12
C114	1977	758	360	305.9	16
D37	1969	591	250	159.9	16
D38	1969	407	340	294.7	16
D47	1963	583	250	182.1	12
D48	1962	556	250	184.5	12

* Information obtained from City of Tucson Water Department.

Table 2. (continued) Well Site Data of 19 Tucson Wells*

Well	Date Drilled	Discharge (gpm)	Bowl Depth (ft)	Pumping Level (ft)	Casing Diameter (in)
E4	1964	236	400	323.5	12
E14	--	299	440	390.2	12
E15	--	416	390	352.9	12
E16	--	405	420	354.6	12
SC6	1954	979	250	172.1	16
SC10	1960	966	190	137.0	16
SS18	1950	698	160	128.8	16
SU	1937	200	453	180	20
MtSt	1966	350	546	180	18

* Information obtained from City of Tucson Water Department

Table 3. Chemical and Physical Analyses of 19 Well Water Samples

Well	pH	turbidity (NTU)	sulfate (mg/L)	nitrate (mg/L)	ammonia (mg/L)	magnesium hardness (mg/L)
B3	7.87	Ø.64	55	4.4	Ø.Ø976	7Ø
B42	7.85	1.4	<5Ø	8.8	Ø.Ø366	12
B45	7.17	1.2	<5Ø	6.6	Ø.Ø61Ø	18
B86	7.72	1.5	7Ø	5.72	Ø.Ø366	36
C48	7.9Ø	1.1	<5Ø	6.6	Ø.Ø854	54
C49	7.72	2.Ø	<5Ø	6.6	Ø	22
C56	7.37	Ø.95	<5Ø	7.48	Ø.Ø61	28
C76	7.22	1.Ø	62	13.2	Ø.3Ø5	4Ø
C87	6.92	1.2	55	11.Ø	Ø.183	38
C114	6.8Ø	Ø.8	<5Ø	6.6	Ø.Ø61	24
D37	7.58	2.2	6Ø	7.48	Ø.Ø61	8Ø
D38	7.63	1.4	<5Ø	11.Ø	Ø	52
D47	7.83	1.Ø	5Ø	5.28	Ø	16
E14	7.87	1.6	<5Ø	8.8	Ø	36
E15	7.8Ø	1.Ø	<5Ø	11.Ø	Ø.122	1Ø
E16	7.75	1.6	<5Ø	7.92	Ø	14
SC6	7.67	1.4	75	7.92	Ø.183	14
SC1Ø	7.35	1.4	9Ø	14.Ø8	Ø.2684	56
SS18	7.55	1.5	2ØØ	13.2	Ø.122	46

Table 3. (continued) Physical and Chemical Analyses of 19 Well Water Samples

Well	iron (mg/L)	calcium hardness (mg/L)	total hardness (mg/L)	total dissolved solids (mg/L)	bacteria log (cfu/ml)
B3	Ø.Ø6	1ØØ	17Ø	175	1.5315
B42	Ø.Ø4	1Ø6	118	9Ø	2.6Ø21
B45	Ø.Ø6	124	142	11Ø	1.5911
B86	Ø.Ø5	148	184	225	1.6232
C48	Ø.Ø7	88	142	12Ø	1.6532
C49	Ø.Ø5	1Ø8	13Ø	135	1.Ø792
C56	Ø.Ø6	116	144	15Ø	1.1761
C76	Ø.Ø3	148	188	16Ø	1.5682
C87	Ø	126	164	16Ø	1.8751
C114	Ø.Ø3	1Ø8	132	15Ø	Ø.9542
D37	Ø.Ø3	1Ø4	184	12Ø	1.1139
D38	Ø.Ø5	94	146	125	2.Ø453
D47	Ø.Ø4	128	144	16Ø	1.7853
E14	Ø.1	11Ø	146	13Ø	1.5798
E15	Ø.Ø3	162	172	135	1.8633
E16	Ø.Ø1	128	142	13Ø	1.Ø862
SC6	Ø.Ø7	212	226	25Ø	Ø.9542
SC1Ø	Ø	228	284	225	1.5315
SS18	Ø.Ø7	312	358	45Ø	Ø.8451

Table 4. Identification of Well Water Isolates

Organism Isolated	WELL #1 cfu	WELL #1 %	WELL #2 cfu	WELL #2 %
Acinetobacter spp.	183	70.4	95	37.4
PO*	25	9.6	36	14.2
Moraxella sp.	7	2.7	38	14.9
Flavobacterium sp.	9	3.4	29	11.4
Pseudomonas/Alcaligenes	1	0.4	3	1.2
Gram-positive rods	13	5.0	17	6.7
Gram-positive cocci	3	1.2	4	1.6
No growth on subculture	19	7.3	32	12.6
Total number of cfu	260		254	
Mean cfu/ml	236		508	
% Gram-negative rods	86.5		79.1	

* PO = pigmented organism

Table 5. Organisms Isolated from WELL #1

Organism	Collection										
	421	513	516	520	525	527	601	603	713	719	725
Acinetobacter spp.	62	2	8	37	3	54	3	4	4	2	4
PO*	-	1	2	10	4	4	-	-	3	-	1
Moraxella sp.	2	1	2	-	-	1	-	-	1	-	-
Flavobacterium sp.	2	-	-	-	3	2	-	-	-	1	1
Pseudomonas/ Alcaligenes	1	-	-	-	-	-	-	-	-	-	-
diphtheroids	-	-	1	4	1	-	1	-	-	-	1
gram-positive rods	-	-	1	2	1	-	1	-	-	-	-
gram-positive cocci	-	-	-	-	2	1	-	-	-	-	-
No growth on subculture	2	1	6	3	-	1	1	1	2	-	2
Total	69	5	20	56	14	63	6	5	10	3	9

* PO = pigmented organism

Table 6. Organisms Isolated from WELL #2

Organism	Collection 520	523	531	712	728
Acinetobacter spp.	19	19	12	19	26
PO*	6	9	5	5	11
Moraxella sp.	4	-	-	25	9
Flavobacterium sp.	10	5	5	3	6
Pseudomonas/ Alcaligenes	-	2	-	1	-
diphtheroids	2	2	3	-	3
gram-positive rods	3	1	-	2	1
gram-positive cocci	1	2	1	-	-
No growth on subculture	7	3	-	9	13
Total	52	43	26	64	69

* PO = pigmented organism

Comparison of Bacterial Numbers Isolated on R2A and SMA

An average of 310 cfu/ml were enumerated on R2A while 118 cfu/ml were enumerated from duplicate water samples plated to SMA (Table 7). There was a significant difference in the number of bacteria/ml well water isolated on the two media (p=0.05).

Identification of Isolates

A total of 514 organisms were isolated for identification. Identification of the isolates revealed the predominace of Gram-negative rods with a limited number of genera represented (Table 4). Members of the genus Acinetobacter predominated (Well #1, 70.4% and Well #2, 37.4%). An oxidase negative, yellow to yellow-orange pigmented, non-motile, Gram-negative rod (PO), not identified by the Lassen method, was consistantly isolated (Well #1, 9.6% and Well #2, 14.2%) (Tables 4,5 and 6). Isolates identified as Moraxella spp. were sporadically isolated from Well #2 and numbers were highest at collection 712 and 728 (Table 7). Moraxella spp. were infrequently isolated from Well #1 (Tables 4 and 5) comprising only 2.7% of the total isolates recovered. In contrast, 14.9% of isolates from Well#2 were identified as Moraxella spp (Table 4 and 6). Flavobacterium spp. were also enriched in Well #2 (Table 4 and 6). Gram-positive organisms comprised a minor percentage of the total isolates (Well #1,

Table 7. Comparison of Heterotrophic Plate Count (HPC) on R2A and SMA media.

		HPC (cfu/ml)	
		R2A	SMA
Collection			
WELL #1	421	690	290
	713	100	10
	719	30	20
	725	90	30
WELL # 2	712	640	240
Total		1550	590

6.2% and Well #2, 8.3%, Table 4). Approximately 7.3% of the total number of bacteria isolated from Well #1 and 12.6% of bacteria isolated from Well #2 did not grow when subcultured to R2A (Tables 4, 5 and 6).

There was no significant difference in the percentage of each genera isolated on either the R2A medium or the SMA (Table 8).

Colony Diameter

There was no significant difference in the diameter of 240 colonies measured on R2A or on SMA ($p=0.05$) (Table 9). Greater than 81% of the diameter of *Acinetobacter* sp. colonies were $\leq$2mm (Table 11). The majority of the *Moraxella* and diphtheroid isolates also were less than 2 mm in diameter (Table 10).

Greater than 63% of the isolates that did not subculture were less than 1 mm in diameter on the primary isolation plates and $\geq$89% were less than 2mm (Table 10).

Bacterial Growth Studies

Representative isolates of the predominate genera grew rapidly in FW and population densities remained high over the 10 day incubation period (Table 11 and Figure 1). There was no significant difference in the growth between individual isolates in the FW.

Table 8. Comparison of the Identification of Ground Water Isolates on R2A and SMA media.

	WELL #2			
	712		728	
	R2A	SMA	R2A	SMA
Acinetobacter spp.	27	7	22	14
PO*	5	-	1Ø	2
Moraxella sp.	25	9	9	3
Flavobacterium sp.	3	-	6	1
Pseudomonas/ Alcaligenes	1	-	-	-
diphtheroids	-	-	3	8
gram-positive rods	2	-	1	-
gram-positive cocci	-	3	-	-
No growth on subculture	9	5	13	1
Total	72	24	64	29

* PO = pigmented organism

Table 9. Colony diameter of Ground Water isolates on R2A and SMA media.

	Colony Diameter					
	<1 mm		1-2 mm		>2 mm	
	R2A	SMA	R2A	SMA	R2A	SMA
Acinetobacter spp.	21	1Ø	8Ø	8	17	3
PO*	12	-	7	2	6	-
Moraxella sp.	1	11	2	-	1	2
Flavobacterium sp.	2	-	-	1	6	-
Pseudomonas/ Alcaligenes	-	-	-	-	-	-
gram-positive rods	1	-	2	-	2	-
gram-positive cocci	2	-	1	-	-	3
No growth on subculture	11	4	3	2	1	-
Total	54/186	26/54	97/186	15/54	35/186	13/54
%	29	48	52	28	19	24

* PO = pigmented organism

Table 10. Distribution of Ground Water isolates on R2A medium by colony diameter.

	Colony Diameter <1 mm		1-2 mm		>2 mm	
	cfu	%	cfu	%	cfu	%
Acinetobacter spp.	54	25.35	119	55.87	40	18.78
PO*	26	42.62	21	34.43	14	22.95
Moraxella sp.	8	19.05	27	64.29	7	16.67
Flavobacterium sp.	12	32.43	12	32.43	13	35.14
Pseudomonas/ Alcaligenes	-	-	2	66.67	1	33.33
diphtheroids	11	61.11	5	27.78	2	11.11
gram-positive rods	3	25.00	6	50.00	3	25.00
gram-positive cocci	2	28.57	1	14.29	4	57.14
No growth on subculture	30	63.83	12	25.53	5	10.64

* PO = pigmented organism

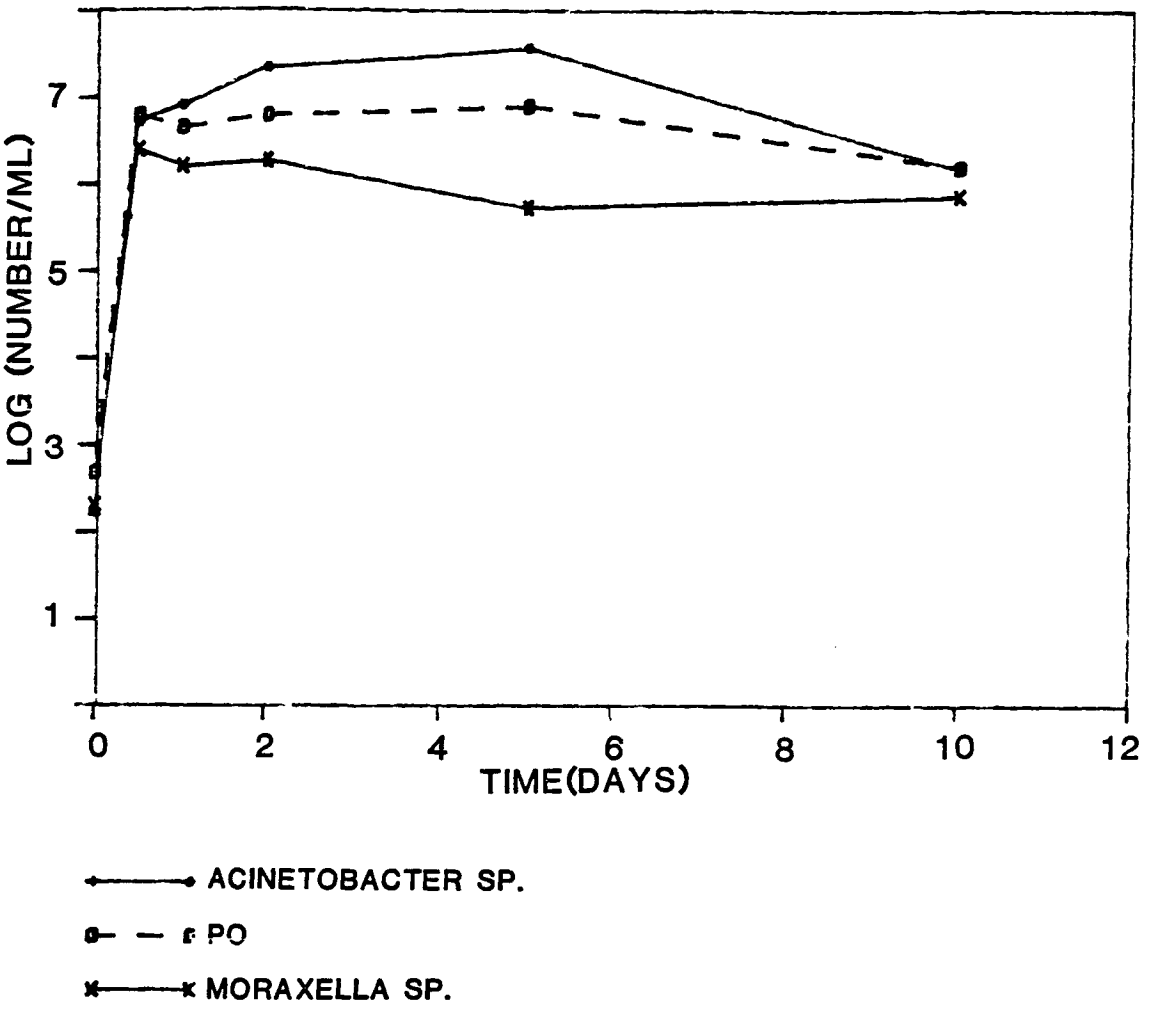
Table 11. Growth of Isolates in Filtered Well Water

	Acinetobacter spp.	PO*	*Moraxella* sp
Time (hours)			
0	2.3138[1]	2.1976	2.6857
12	6.3964	6.7118	6.7979
24	6.2087	6.9119	6.6573
48	6.2719	7.3367	6.6573
120	5.7385	7.5761	6.9065
240	5.8771	6.1822	6.2045

PO* = Pigmented Organism

1 = Log_{10} Acridine Orange Direct Count/ml Well Water

FIGURE 1



GROWTH OF BACTERIAL ISOLATES IN FILTERED WELL WATER

Growth of the Acinetobacter sp. and PO was stimulated by the addition of low concentrations of organic carbon (Table 12 and 13, and Figure 2). In contrast, growth of the Moraxella sp. was not stimulated above that obtained in FW (Table 14 and Figure 2).

HPC and AODC were similar (Table 15) indicating that bacteria counted microscopically were indeed viable.

Survival Studies of Bacteria in Well Water

Bacterial numbers demonstrated a greater than 3 orders of magnitude increase within 24 hours of incubation at in situ temperature in all 19 well waters tested (Table 16 and 17). In samples from 17 of the 19 wells tested maximum bacterial numbers were achieved after 2-3 days incubation. Maximum growth in Well B45 was attained after 7d and Well SS18 after 5d incubation. Bacterial numbers remained high and decreased Ø.36-1.8 orders of magnitude after 3Ø days incubation (Table 17). With the exception of sample E14, there was no significant difference between AODC and HPC (Table 18).

Approximately 72% of the bacterial isolates from samples of the 19 wells were Gram-negative rods (Table 19).

Table 12. Effect of Organic-Carbon Enrichment on Growth of *Acinetobacter* isolate in Filtered Well Water

Substrate	Concentration (ug carbon per liter)	Time (hours) 24	48	120	240
Glucose	100	1.2776[1]	1.2931	2.3649	2.8620
	1000	1.1206	1.3669	2.4477	2.2826
Pyruvate	100	1.4132	1.0824	2.5762	1.8457
	1000	1.6931	1.3322	2.3818	1.9280
Acetate	100	1.7707	1.9409	2.5367	2.2218
	1000	1.4343	1.3389	2.4909	1.9623
Succinate	100	1.2984	1.2179	2.5028	2.2450
	1000	0.9155	1.4605	2.4726	1.9287

1 = (Log_{10} Acridine Orange Direct Count (AODC)/ml substrate enriched Filtered Water) - (Log_{10} AODC/ml Filtered Water)

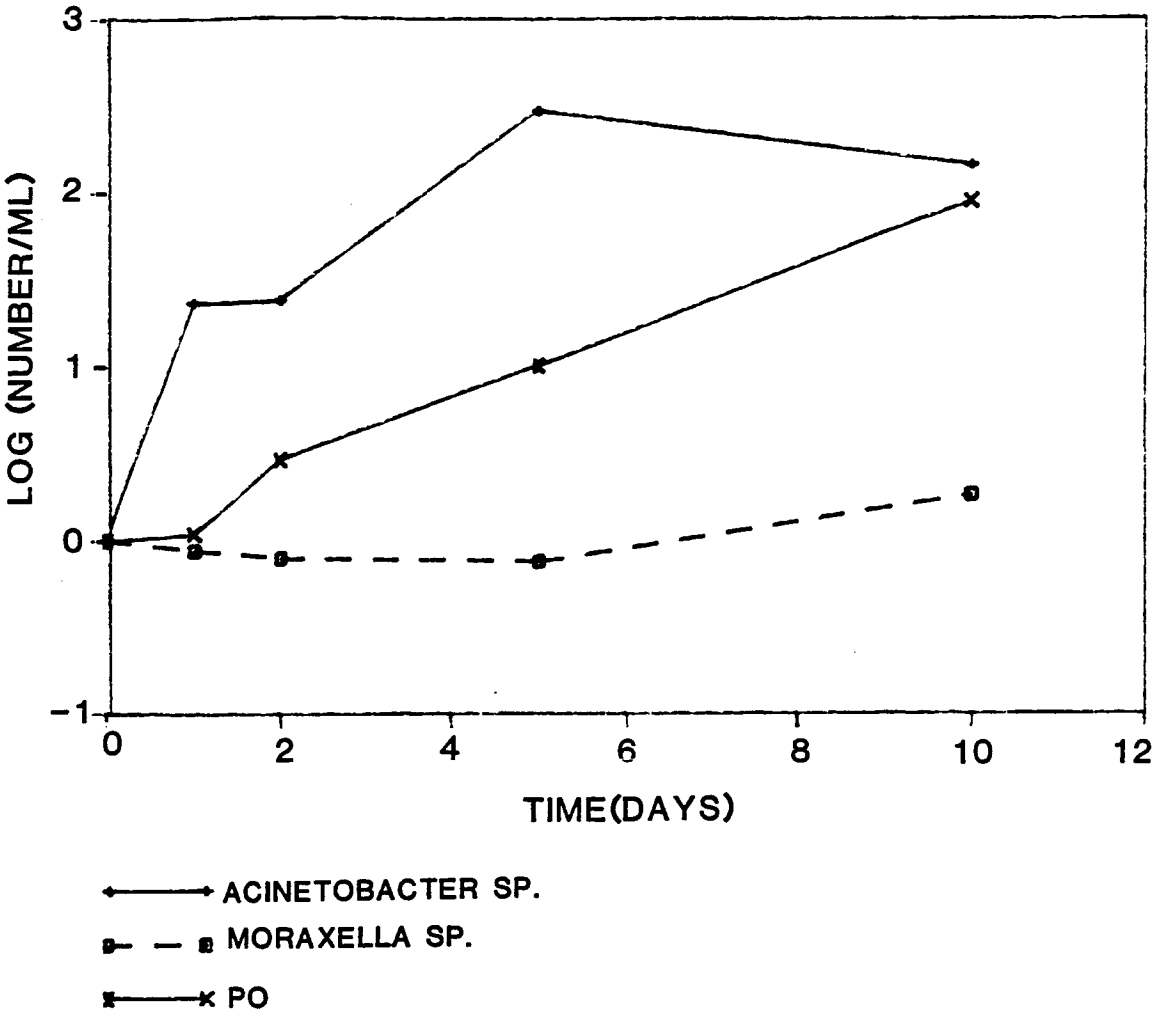
Table 13. Effect of Organic-Carbon Enrichment on Growth of Isolate PO* in Well Water

Substrate	Concentration (ug carbon per liter)	Time (hours) 24	48	120	240
Glucose	100	-0.0034[1]	0.3382	1.2382	2.4923
	1000	0.2001	0.4612	0.8485	2.0039
Pyruvate	100	0.2515	0.5948	1.0370	1.4702
	1000	0.0285	0.3643	1.1228	2.0390
Acetate	100	0.3174	0.6138	0.8542	1.5602
	1000	-0.3775	0.6135	0.8497	2.6706
Succinate	100	-0.3815	0.4699	1.1095	1.5830
	1000	0.2943	0.4699	1.1095	1.8121

*PO = pigmented organism

1 = (Log_{10} Acridine Orange Direct Count(AODC)/ml substrate enriched Filtered Water) - (Log_{10} AODC/ml Filtered Water)

FIGURE 2



GROWTH OF BACTERIAL ISOLATES IN CARBON-ENRICHED WELL WATER

Table 14. Effect of Organic-Carbon Enrichment on Growth of *Moraxella* Isolate in Well Water

Substrate	Concentration (ug carbon per liter)	Time (hours) 24	48	120	240
Glucose	100	-0.1966 [1]	-0.0726	-0.0957	0.2814
	1000	-0.1310	-0.0524	-0.0957	0.2814
Pyruvate	100	0.0566	-0.0644	-0.0223	0.6598
	1000	-0.0354	-0.0767	-0.0186	0.5399
Acetate	100	-0.2444	-0.0524	-0.0020	0.1064
	1000	-0.3071	-0.0894	-0.1674	0.2209
Succinate	100	0.5883	-0.3120	-0.2846	0.4856
	1000	-0.2004	-0.0938	-0.1947	-0.2814

1 = (Log_{10} Acridine Orange Direct Count (AODC)/ml substrate enriched Filtered Water) - (Log_{10} AODC/ml Filtered Water)

Table 15. Comparison of Direct Microscopic Count (AODC) to Viable Heterotrophic Plate Count (HPC) in the Enumeration of Ground Water Isolates

		Time (hours)			
		Ø	24	48	12Ø
Isolate	Method				
Acinetobacter	AODC	-	5.8642[1]	6.4588	5.6672
	HPC	2.1789[2]	>6.0000	5.4771	5.2989
PO*	AODC	-	5.4232	6.756Ø	6.4917
	HPC	2.4886	>6.0000	4.3Ø1Ø	5.7243
Moraxella	AODC	-	6.41Ø8	6.4774	6.7522
	HPC	2.3222	>6.0000	6.1761	5.1761
Flavobacterium	AODC	-	6.Ø128	6.63Ø5	6.4569
	HPC	2.Ø6Ø7	>6.0000	6.0934	5.4314

*PO = pigmented organism

1 = $Log_{1Ø}$ Acridine Orange Direct Count/ml Filtered Water

2 = $Log_{1Ø}$ cfu/ml

Table 16. Acridine Orange Direct Count (AODC) of Bacteria in Well Water

Time (Days)	WELL			
	B3	B42	B45	B86
Ø	2.5315[1]	3.6Ø21	2.5911	2.6232
1	6.4ØØØ	6.8771	5.7373	6.4649
2	6.8273	6.8579	7.4645	7.ØØ87
3	7.4522	7.5863	7.4882	7.2533
5	6.9257	7.1782	7.3598	7.Ø969
7	6.985Ø	7.1547	7.514Ø	7.Ø668
1Ø	6.5571	6.52Ø2	7.1981	6.4117
16	6.3941	6.4479	6.95Ø2	6.4191
21	6.4458	6.95Ø2	7.1634	6.5263
3Ø	6.45Ø1	6.41Ø7	7.1521	6.4628

1 = $Log_{1Ø}$ Bacteria/ml Well Water

Table 16. (continued) Acridine Orange Direct Count (AODC) of Bacteria in Well Water

Time (Days)	WELL				
	C48	C49	C56	C76	C87
Ø	2.6532[1]	2.Ø792	2.1761	2.5682	2.8751
1	5.8814	5.8288	5.6494	6.36Ø8	6.2561
2	6.8ØØ1	6.8171	6.7365	7.1836	6.798Ø
3	7.4294	7.4586	7.3114	7.2246	7.5385
5	7.1721	7.3819	7.1822	7.Ø144	7.Ø368
7	6.4991	7.1142	6.9782	7.15Ø1	7.2357
1Ø	6.3815	6.5284	6.32Ø8	6.342Ø	6.4989
16	6.4969	6.5243	6.3672	6.7274	6.4323
21	6.4544	6.4212	6.46Ø7	6.5923	6.1Ø33
3Ø	6.4479	6.3473	6.4479	6.6633	6.3583

1 = $Log_{1Ø}$ Bacteria/ml Well Water

Table 16. (continued) Acridine Orange Direct Count (AODC) of Bacteria in Well Water

Time (days)	WELL				
	C114	D37	D38	D47	E14
Ø	1.9542[1]	2.1139	3.Ø453	2.7853	2.5798
1	5.2751	5.9119	6.2861	6.26Ø4	6.2492
2	6.9543	7.1521	6.9272	7.3152	7.1959
3	7.624Ø	7.9583	7.6347	7.4ØØØ	8.Ø163
5	7.2Ø87	7.ØØ21	7.2322	7.2513	7.14Ø4
7	6.4396	7.Ø91Ø	7.1424	7.Ø22Ø	6.8897
1Ø	6.4569	6.9759	6.8413	6.4434	6.2625
16	6.3622	6.7541	6.4Ø22	6.4354	6.3672
21	6.6483	6.4626	6.4458	6.2861	6.1715
3Ø	6.4828	6.4378	6.4832	6.6262	6.17Ø9

1 = $Log_{1Ø}$ Bacteria/ml Well Water

Table 16. (continued) Acridine Orange Direct Count (AODC) of Bacteria in Well Water

Time (Days)	WELL				
	E15	E16	SC6	SC10	SS18
0	2.8633[1]	2.8062	1.9542	2.5315	1.8451
1	6.8192	6.1482	6.0778	5.7316	6.3880
2	7.4294	7.3152	6.6807	7.0075	7.1097
3	7.1284	7.7345	7.6633	7.8066	7.1139
5	6.9098	7.1256	7.3031	7.3010	7.5263
7	7.1160	7.2087	7.2451	7.1404	7.3608
10	6.5943	6.2239	7.3909	6.9563	6.6347
16	6.5592	6.3230	6.4911	6.5823	7.4334
21	6.6219	6.0021	6.4719	6.5098	7.1559
30	6.5822	6.2368	6.3252	6.4479	7.1304

1 = Log_{10} Bacteria/ml Well Water

Table 17. Growth of Bacteria from 19 Well Water Samples

WELL	TIME		
	T24-T0[1]	Tmax-T0[2]	Time to Tmax (hours)
B3	3.8685	4.9207	72
B42	3.2750	3.9842	72
B45	3.1462	4.9229	168
B86	3.8417	4.6301	72
C48	3.2282	4.7762	72
C49	3.7496	5.3794	72
C56	3.4733	5.1353	72
C76	3.7926	4.6564	72
C87	3.3810	4.6634	72
C114	3.3209	5.6698	72
D37	3.7980	5.8444	72
D38	3.2408	4.5894	72
D47	3.4751	4.6147	72
E14	3.6694	5.4365	72
E15	3.9559	4.5661	48
E16	3.3420	4.9283	72
SC6	4.1236	5.0791	72
SC10	3.2001	5.2751	72
SS18	4.4529	5.6812	120

1 = Log_{10} Bacteria/ml at time 24 hours - time 0 hours

2 = Log_{10} Bacteria/ml at time maximum bacterial number - time 0 hours

Table 18. Comparison of Bacterial Numbers using Acridine Orange Direct Count (AODC) and Heterotrophic Plate Count (HPC)

	BACTERIAL NUMBERS			
	AODC		HPC	
WELL	T720-T0[1]	Tmax-T720[2]	T720-T0	Tmax-T0
B3	3.9186	1.0021	3.8487	1.0720
B42	2.8086	1.1756	2.8750	1.1092
B45	4.5610	0.3619	4.3174	0.6055
B86	3.8396	0.7905	4.1172	0.5129
C48	3.7947	0.9815	4.4607	0.3155
C49	4.2681	1.1113	4.2218	1.1576
C56	4.2718	0.8635	4.9669	0.1684
C76	4.0951	0.5613	4.0753	0.5811
C87	3.4832	1.1802	4.1072	0.5562
C114	4.6286	1.1412	4.9783	0.7915
D37	4.3239	1.5205	4.8206	1.0238
D38	3.3479	1.1515	4.0116	0.5778
D47	3.8409	0.7738	3.9706	0.6441
E14	3.5911	1.8454	5.1328	0.3037
E15	3.7189	0.8472	4.0175	0.5486
E16	3.4306	1.4977	4.6154	0.3129
SC6	4.3710	1.3381	4.7534	0.9557
SC10	3.9164	1.3587	4.3493	0.9258
SS18	5.2853	0.3959	5.3480	0.3332

1 = Log_{10} Bacterial Number (Time 720 hours) - (Time 0)

2 = Log_{10} Bacterial Number (Time at maximum number) - (Time 720 hours)

Table 19. Characteristics of Bacteria Isolated From 19 Well Water Samples

Well	Colony Morphology	Gram Reaction	Oxidase
B3,C48,C49,C76, D37,E14,SC6	large yellow	- rod	+
B3,B86,C76	orange	- rod	+
B3,D37	pink	- rod	+
B3,B45,D47,E15	small yellow	- rod	-
B3,B42,B45,B86, C48,C87,C114,D37, D38,E14,E15,E16, SC6	small grey	- rod	-
B3,C76,D47,SC10, SC10,SS18	cream	+ diphtheroid	
B42	white	+ cocci	
B45,C56,C114	raised pink	+ rod	
B45,D38	small grey	+ diphtheroid	
B86	large yellow	- rod	-
B86,C48,C49,C56 D37	flat grey	- rod	+
B86,C114,SS18	small yellow	+ diphtheroid	
C48,C56,D38,E14, E16,SC10	cream	- rod	+

Table 19. (continued) Characteristics of Bacteria Isolated From 19 Well Water Samples

Well	Colony Morphology	Gram Reaction	Oxidase
C49,C56,D38	cream	- rod	-
C76,D37,SC1Ø	raised yellow	- rod	+
C87,C114,E15	cream	+ rod	
C87,D47,E16	raised pink	+ diphtheroid	
C114,D37,SC1Ø	flat yellow	- rod	+
C114	raised white	+ rod	
D37	white	+ diphtheroid	
D38	yellow-brown	- rod	+
D47	pink	+ rod	
E14,E16,SS18	flat grey	- rod	-
E14	large white	- rod	+
E15	cream	+ diphtheroid	

DISCUSSION

The isolation of heterotrophic bacteria from well water was significantly enhanced by the use of a low nutrient medium (R2A) incubated at in situ temperatures. The use of SMA, the standard medium for the enumeration of bacteria in water, therefore may result in lower bacterial counts not truly representative of the microbial numbers present in the well water community.

Colony diameters of bacteria isolated on R2A and SMA were small but similar thus indicating that R2A supplies sufficient nutrient content. Whether small colony size is consistant with decreased cell size as observed by other investigators who have studies the microbiology of dilute environments (MacDonnell and Hood, 1982 and Wilson et al., 1983) is not known. Decreased cell mass however may represent one mechanism necessary for survival in oligotrophic aquatic environments.

The predominance of a limited number of genera in the well waters tested supports the findings of other researchers who have studied unpolluted ground water wells (Willis et al., 1976). Acinetobacter spp. comprised the largest percentage of bacteria isolated. Representatives of the three most common organisms isolated, Acinetobacter sp., PO,and Moraxella sp. grew rapidly in unamended filtered

well water and is an indication of the adaptibility these bacteria to low nutrient well water environments. Stimulation of growth of the Acinetobacter isolate by enrichment of well water with glucose, acetate, succinate, or pyruvate in part demonstrated the ability of this organism to utilize a variety of carbon substrates. This rapid and significant increases in population further enhances their competitive ability for available nutrients. The predominance of Acinetobacter spp. in the well water may reflect the ability of this genus to efficiently utilize nutrients that may be introduced to the aquifer.

Moraxella sp. was able to grow in FW but was not stimulated by the low concentrations of carbon in the growth studies. This lack of competitive advantage may result in the low percentage of Moraxella spp.in the total number of isolates from the wells studied. The sudden sharp increase in the percentage of Moraxella spp. in Well #2 at collection 712 (39.1%) and 728(13.Ø%) may reflect a recharge of the well water after heavy rain as samples 712 and 728 were collected approximately 24-36 hours after heavy rains This well is located down gradient from a water canal that overflows during the heavy summer rains experienced in the desert southwest. Introduction of Moraxella spp. could have occurred at that time.

PO grew in FW but not at the same rate or extent as observed for the Acientobacter isolate (Table 11 and 12, Figure 2).

AODC proved to be a simple, rapid, reliable method for the enumeration of bacteria in water. Since viable bacterial counts (HPC) were statistically equal to those bacterial counts obtained with AODC, cells counted by the later were considered viable. Counting non-viable cells with AODC could have given rise to misleading results in both the FW and carbon amended FW studies and in the 19 well survival studies.

Bacterial survival over the 30 day period was consistant for all 19 wells studied. The increase in microbial numbers in 89% of the wells within 3 days demonstrated the presence of bacterial populations capable of significant growth in situ. The bacterial counts attained in the native well water parallel the results of the isolates' growth in FW. This suggests that the selected isolates probably retained their "natural" growth characteristics after isolation on artifical medium, or reverted to them during serial passage in FW. While bacteria isolated from the 19 wells were not identified, oxidase reactions and colony pigmentation suggested that they were similar to those identified from Wells #1 and #2. Since 61.8% of the Gram-negative isolates from the 19 well

studies were non-pigmented and oxidase negative, the predominance of Acinetobacter spp. is likely.

Growth and maintenance of large numbers of bacteria even after 30 days incubation suggests the possibility that increased numbers of these bacteria could be released into the distribution system. The consistancy in types of organisms isolated from Wells #1 and #2 and those isolated from the 19 survey wells suggests that large numbers of potential pathogens may reside "naturally" in Tucson's drinking water. The ability of Acinetobacter spp. to utilize low concentrations of a variety of carbon compounds which resulting in increased growth reflects the ability of these bacteria to not only exist as transitory or "naturally occurring" organisms but to survive and establish resident populations in the water system.

The presence of significant populations of non-coliform bacteria in well water identified as potential opportunistic pathogens may then impact the overall quality of untreated drinking water.

APPENDIX

R2A Medium

Ingredient	Concentration (grams/liter)
Yeast Extract	Ø.5
Proteose Peptone No. 3	Ø.5
Casamino Acids	Ø.5
Glucose	Ø.5
Soluble Starch	Ø.5
Sodium Pyruvate	Ø.3
K_2HPO_4	Ø.3
$MgSO_4$	Ø.Ø5
Agar	15.Ø

Final pH 7.2, adjust with K_2HPO_4 or KH_2PO_4 before adding agar. Add agar, heat medium to boiling to dissolve agar and autoclave for 15 minutes, at 121 C, 15 psi.

REFERENCES

Akagi, Y., N. Taga, and U. Simidu. 1977. Isolation and distribution of oligotrophic marine bacteria. Can. J. Microbiol. 23:981-987.

Akagi, Y., U. Simidu, and N. Taga. 1980. Growth responses of oligotrophic and heterotrophic marine bacteria in various substrate concentrations, and taxonomic studies on them. Can. J. Microbiol. 26:800-806.

American Public Health Association. 1980. Standard Methods for the Enumeration of Water and Waste Water, 15 ed., Am. Public Health Assoc., Washington, D.C.

Bitton, G. and C.P. Gerba. 1984. Groundwater Pollution Microbiology. John Wiley and Sons, New York.

Bott, T.L. and T.D. Brock. 1970. Growth and metabolism of periphytic bacteria:methodology. Limnol. Oceanogr. 15:333-341.

Brock, T.D. 1978. Use of fluorescent microscopy for quantifying phytoplankton, especially filamentous blue-green algae. Limnol. Oceanogr. 23:158-160.

Buchanan, R.E. and N. E. Gibbons. 1974. Bergey's Manual of Determinative Bacteriology, 8th ed. Williams and Wilkins, Baltimore, Maryland.

Carson, L. A., M.S. Favero, W.W. Boyd, and N.J. Petersen. 1973. Morphological,biochemical and growth characteristics of Pseudomonas cepacia from distilled water. Appl. Microbiol. 25:476-483.

Daley, R.J. and J.E. Hobbie. 1975. Direct counts of aquatic bacteria by a modified epi-fluorescent technique. Limnol. Oceanogr. 20:875-882.

Druilhet, R.E. and J.M. Sobek. 1976. Starvation survival of Salmonella enteritidis. J. Bacteriol 125:119-124.

Freeze, R.A. and J.A. Cherry. 1979. Groundwater. Prentice-Hall inc., Englewood Cliffs, New Jersey.

Geldreich, E.E., M.J. Allen, and R.H. Taylor. 1978. Interferences to coliform detection in potable water supplies. In C.W. Hendricks (ed.), Evaluation of the Microbiology Standards for Drinking Water. U.S.Environmental Protection Agency, Washington,D.C.

Hagstrom, A., U. Larson, P. Horstedt, and S. Normark. 1979. Frequency of dividing cells, a new approach to the determination of bacterial growth rates in aquatic environments. Appl.Environ.Microbiol. 37:805-812.

Hendricksen, S.D. 1973. Moraxella, Acinetobacter, and the Mimeae. Bacteriol. Rev. 37:522-561.

Henrici, A. T. 1933. Studies of freshwater bacteria. I. A direct microscopic technique. J. Bacteriol. 25:277-286.

Henrici, A.T. and D.A. Johnson. 1935. Studies of freshwater bacteria. II. Stalked bacteria, a new order Schizomycetes. J. Bacteriol. 30:324-361.

Herson, D.S. and H.T. Victoreen. 1980. Hinderence of coliform recovery by turbidity and non-coliforms. U.S. Environmental Protection Agency, Cincinnati, Ohio.

Hobbie, J.E., R. J. Daley, and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225-1228.

Hutchinson, D., R.H. Weaver, and M. Scherago. 1943.The incidence and significance of microorganisms antagonistic to Escherichia coli in water. J. Bacteriol. 45:29.

Jannasch,H.W. 1967a. Enrichments of aquatic bacteria in continous culture. Arck. Mikrobiol. 59:165-173.

Jannasch, H.W. 1967b.Growth of marine bacteria at limiting concentrations of organic carbon in seawater. Limnol. Oceanogr. 12:264-271.

Krane, S.A. and G.J. Sutter. 1962. Statistical methods for assuring precision of microscopic counts. U.S. Army Chemical Corps. Reference Note 6-62-1. C-E-I-R,Inc., Dugway Field Operations, Dugway, Utah.

Kuznetsov, S.I., G.A. Dubinina, and N.A. Lapteva. 1979. Biology of oligotrophic bacteria. Ann. Rev. Microbiol. 33:377-387.

Lamka, K., M.M. LeChavallier, and R,J, Seidler. 1980. Bacterial contamination of drinking water in a modern rural neighborhood. App. Environ. Microbiol. 39:734-738.

Larson, R. and J.L. Pate. 1975. Growth and morphology of Asticcacaulis biprosthecium in defined media. Arch. Microbiol. 106:147-157.

Lassen, J. 1975. Rapid identifiaction of Gram-negative rods using a three-tube method combined with a dichotomic key. Acta. Path. Microbiol. Scand. Sect. B. 83:525-533.

MacDonell, M.T. and M.A. Hood. 1982. Isolation and characterization of ultramicrobacterium from a Gulf Coast estuary. Appl. Environ. Microbiol. 43:566-571.

MacFaddin, J.F. 1980. Biochemical Tests for Identification of Medical Bacteria, 2nd ed. Williams and Wilkins, Baltimore, Maryland.

Matthess, G. 1982. The Properties of Groundwater. John Wiley and Sons, New York.

McNabb, J.F. and W.J. Dunlap. 1975. Subsurface biological activity in relation to ground-water pollution. Groundwater. 13:33-44.

Murray, C.R. and E.B. Reeves. 1972. Estimated use of water in the United States in 1970. U.S. Geological Survey Circ. 676.

Novitsky, J.A. and R.Y. Morita. 1976. Morphological characterization of small cells resulting from nutrient starvation of a psychrophilic marine vibrio. Appl. Environ. Microbiol. 32:617-622.

Novitsky, J.A. and R.Y. Morita. 1978. Possible strategy for the survival of marine bacteria under starvation conditions. Marine Biology. 48:289-295.

Pedros-Alio, C. and T.D. Brock. 1982. Assessing biomass and production of bacteria in eutrophic Lake Mendota, Wisconsin. Appl. Environ. Microbiol. 44:203-218.

Poindexter, J.S. 1981. The Caulobacters: ubiquitous unusual bacteria. Microbiol. Rev. 45:123-179.

Reasoner, D.J. and E.E. Geldreich. 1979. A new medium for the enumeration and subculture of bacteria from potable water. Proceedings of the 79th Annual Meeting of the American Society for Microbiology.

Scalf, M.R., J.F. McNabb, W.J. Dunlap, R.L. Cosby, and J. Fryberger. 1981. Manual of Ground-Water Quality Sampling Procedures. Nat. Well Water Assoc. Worthington, Ohio.

Todd, D.K. 1980. Groundwater Hydrology, 2ed. John Wiley and Sons, New York.

van der Klooij, D., A. Visser, and W.A.M. Hijnen. 1980. Growth of Aeromonas hydrophilia at low concentrations of substrates added to tap water. Appl. Environ. Microbiol. 39:1198-1204.

van der Klooij, D. and W.A.M. Kijnen. 1981. Utilization of low concentrations of starch by a Flavobacterium species isolated from tap water. Appl. Environ. Microbiol. 41:216-221.

Waksman, S.A. 1916. Bacterial numbers in soil, at different depths, and in different seasons of the year. Soil Science. 1:363-380.

Willis, C.J., G.H. Elkan, E. Horvath, and K.R. Dail. 1976. Bacterial flora of saline aquifers. Groundwater. 13:406-409.

Wilson, J.T., J.F. McNabb, D.L. Balkwell, and W.C. Ghiorse. 1983. Enumeration and characterization of bacteria indigenous to a shallow water-table aquifer. Groundwater. 21:134-142.

Zaporozec, A. 1972. Graphical interpretation of water quality data. Groundwater. 10. 32-43.

ZoBell, C.E. and C.W. Grant. 1942. Bacterial activity in diltute nutrient solutions. Science. 96:189.