2000

Evaluation of survival and recovery characteristics of bifidobacteria as indicators of fecal pollution of water

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EVALUATION OF SURVIVAL AND RECOVERY CHARACTERISTICS OF BIFIDOBACTERIA AS INDICATORS OF FECAL POLLUTION OF WATER

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Thesis submitted to the College of Agriculture, Forestry, and Consumer Sciences at West Virginia University
in partial fulfillment of the requirements
for the degree of

Master of Science
in
Plant and Soil Science
with a concentration in Environmental Microbiology

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Morgantown, West Virginia
2000

Keywords: Bifidobacteria, Indicator organisms, Fecal pollution

ABSTRACT

EVALUATION OF SURVIVAL AND RECOVERY CHARACTERISTICS OF BIFIDOBACTERIA AS INDICATORS OF FECAL POLLUTION OF WATER

John Elmer Ravenscroft, Jr.

The examination of aquatic environments for bacterial indicator organisms has proven to be a useful and well-established practice for the purpose of monitoring microbiological water quality. An ideal indicator of fecal pollution should be present in sufficient density to allow detection, present simultaneous with pathogen(s), incapable of aftergrowth in external aquatic environments, easy to enumerate, and exclusively of fecal origin. Unfortunately, none of the indicator organisms presently in use today meet all of these requirements. Bifidobacteria show promise as indicators of fecal pollution in water with the additional ability of potentially distinguishing between fecal pollution of human and animal origin. In laboratory microcosm experiments, the survival of the bifidobacteria population was indirectly proportional to the temperature at which the microcosm was stored. In mixed microcosm studies with *E. coli*, the survival of the bifidobacteria population was considerably less than the *E. coli* population. The recovery of bifidobacteria from constructed wetlands receiving primary treated sewage was monitored using published selective media (YN6, BIM25, and BIM50 agar). In the influent and wetland samples, there was approximately a 2 to 3 log reduction of bifidobacteria, while fecal coliforms exhibited a 4 to 5 log reduction. Results indicate the YN6 medium lacks the desired sensitivity and selectivity to effectively enumerate bifidobacteria. Studies addressing the recovery of bifidobacteria from the wetlands with modified bifidobacteria enumeration media were mixed to unsuccessful.

ACKNOWLEDGEMENTS

The dictionary defines the word *mentor* as a wise and trusted counselor or teacher. I consider myself quite fortunate that in collegiate pursuits, I have had wise counsel from two trusted colleagues that I would like to properly thank in this text. First, I would like to acknowledge the guidance and encouragement imparted to me by Anthony Slavinski. I worked as a student intern in the same division as Mr. Slavinski, a registered sanitarian, at the Allegany County Health Department in Cumberland, Maryland. Recognizing my desire to further pursue my love of science, Mr. Slavinski selflessly encouraged and prodded me to apply to graduate school. He was always a source of sage advice and assistance whenever I needed. His wise counsel continues to make my graduate career a rewarding experience. Secondly, I would like to express a great deal of appreciation to my advisor, Dr. Gary K. Bissonnette. He has embodied the meaning of the word mentor; coupling professionalism and kindliness into a truly rare combination. His guidance transformed my inexperience and anxiety at the beginning of my graduate program into confidence that I feel to this day. He was always approachable and accessible when I had a question, a problem, or comment. I feel very fortunate to have worked with him and should I find myself mentoring students someday, I would do my best to emulate his example.

Many thanks go out to my graduate committee, Drs. Alan Sexstone, Daniel Panaccione, and Henry Rauch for their input and patience during this project. A special note of gratitude goes to Dr. Sexstone for his assistance in furthering my graduate career

at the doctoral level. I would also like to thank Dr. Joseph P. Calabrese for his help in designing the anaerobic flasks and his advice during those occasional ‘rough spots’ in the course this project.

A deep and sincere thanks to my parents, John and Shirley, for their unfettered love and support throughout my life. Their kind words and encouragement helped me keep the faith and finish what I started. I would also like to express my appreciation to my sister Melissa, who having gone through graduate school herself has always been instrumental in helping me maintain my perspective.

I would also like to thank the many friends and co-workers who made my time at West Virginia University a pleasant and memorable experience.

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LITERATURE REVIEW

Water is seldom found in a pure state in nature whether it be in the form of surface or ground water. The quality of water that we ingest is a critical parameter in determining the overall quality of our lives (20). Of particular concern to public health is the contamination of freshwater supplies by pathogenic organisms. One of the most dangerous forms of contamination occurs when feces from animals or humans enter the water supply. This can result in the spread of disease when a pathogen is shed in feces, contaminates a water supply, and is later ingested. However, not all pathogens need to be ingested, some helminths bore directly through the skin and are spread among persons who swim or wade in waters contaminated by human wastes. This review will address concerns for monitoring both surface and ground water, presently accepted standards for determining the quality of these supplies, and a proposal for a supplemental alternative for better evaluating water supply quality.

Ground water quality. Ground water has been defined as the water contained in interconnected void spaces located below the water table in either an unconfined aquifer or a confined aquifer (20). Unconfined aquifers are usually located near the land surface and are easily recharged. They are also known as water table aquifers because the water table is its upper boundary. Confined aquifers which are totally filled with ground water, occur further below the ground surface. They form between low permeability layers of rock or sediments and are consequently under constant

pressure from the overlying stratum. The water level in a well tapping only a confined aquifer will be above the top of that aquifer (1). Ground water forms from excess soil moisture being pulled downward by gravity until it reaches a saturated zone or water table. As the water percolates through the soil, processes such as ion exchange, adsorption, precipitation, filtration, and chemical alteration occur which produce ground water that may differ chemically and microbiologically from surface water (50).

Of the small percentage of the world's total water available as fresh water, more than 98 percent is classified as ground water. This greatly exceeds the amount available as surface water (20). In the United States, approximately 30-60 quadrillion (10^{15}) gallons of ground water exist within 2500 feet vertically of the land surface. This is more than four times the volume of the Great Lakes, 30 times the volume of all fresh water lakes, and 3000 times greater than the volume of all streams in the U.S. (1,40). The usable ground water resources of the U.S. are equivalent to 35 times the total annual surface runoff or about 400 times the total annual consumption of water (54).

There is a popular misconception that ground water is a reliable source of good quality drinking water. This misconception stems from the fact that surface water pollution frequently results in contamination that can be detected by sight or smell, whereas ground water pollution often cannot be detected by the human senses alone (20). Microbes, such as bacteria, protozoa, viruses, and fungi; gases, such as methane and carbon dioxide; inorganic material, such as metals and salts; and organic matter are all integral components of natural ground water (1). Varying

concentrations of the different constituents all contribute to the overall quality of a ground water source. However, an intimate knowledge of microbiology or chemistry is not needed to understand the potential health hazards associated with consuming contaminated water. For example, Dr. John Snow in 1854 first linked the contamination of wells by cholera to discharge and percolation from earthen privy vaults prior to the discovery of the causative organism (5). By simply breaking a pump handle and thus closing a widely used well, he ended an epidemic of cholera in London.

Reliance on ground water is quite widespread, with greater than 50 percent of the population of the United States currently receiving their primary drinking water from ground water sources (40,50). Additionally, ground water usage tripled between 1950 and 1980 – from about 30 billion gallons per day to almost 90 billion gallons per day in 1980 (54). This trend is continuing. One-fifth of the fresh water used in the U.S. is furnished by ground water (60). Thirty-six percent of the municipal public drinking water supplies come from ground water and 75 percent of major U.S. cities depend on ground water for most of their supply (40). Ground water also supplies 95 percent of the rural homes in the U.S. with water for domestic use (1). In West Virginia, a U.S. Environmental Protection Agency (USEPA) study indicated that more than 53 percent of the residents rely on ground water as a source of drinking water. Ground water supplied 32 percent of the residents on public supplies and 97 percent of rural residents on private supplies (13).

Although ground water pollution has occurred for centuries, increased industrialization, population density, and agricultural activities have greatly

contributed to the problem (40). Sources of ground water contamination as a result of human activity include a wide range of agricultural, industrial, and municipal inputs. They include subsurface percolation from septic systems, land application of waste water, leachate from landfills, surface impoundments, and illegal dumping to agricultural runoff, induced discharge from mining and drilling operations, and even human and animal burial sites (60). Contamination may occur through point or non-point sources. Point sources are those whose origins can be determined and occur in a limited area, whereas non-point sources are more ambiguous (44). Altogether, substantial amounts of solid, liquid, and gaseous waste are generated in the U.S – as much as 29 metric tons per person per year (54).

Widespread usage coupled with increasing frequency of ground water pollution could result in a potentially dangerous public health problem. Unlike organic contaminants of ground water (e.g., TCE) which result in a long-range public health hazard, microbial contamination results in a more immediate effect, frequently to a large number of people. For example, 28,745 persons in Delhi, India were infected with hepatitis A virus transmitted via consumption of improperly disinfected drinking water (3). A Center for Disease Control study of waterborne disease outbreaks in the U.S. from 1986 to 1988 found 56 percent of the outbreaks linked to well water and 32 percent of the illnesses directly attributed to untreated ground water (29). Another study showed 672 cases of waterborne disease in the U.S. from 1946 to 1980. Untreated ground water was responsible for 35 percent of the outbreaks, eight percent was due to untreated surface waters, and the remainder were caused by failure of systems designed to treat contaminated ground water (20).

Surface and Drinking Water Quality. Recreational water environments and potable water systems can easily become polluted with pathogens from normal, diseased, or carrier human and animal excrements. Potable community, non-community, and private water supplies can be contaminated in many ways, including: cross connections between a water main and a sewer; entry of sewage through leaks in damaged pipes; the air-water interface in the distribution system, e.g. storage tanks; and neglected point-of-use devices (48). Contamination of drinking water supplies can have serious implications for the people these systems serve. For example, between 1971 and 1988, there were 545 waterborne disease outbreaks caused by contaminated water supplies affecting over 136,000 people in the United States (29). Just in the three year period from 1986 to 1988 there were 50 outbreaks of illness from contaminated drinking water affecting almost 26,000 people (29).

The quality of recreational waters, which include bathing beaches, is dependent on the physical, chemical, and biological components of the surface water system. The potential of recreational waters for disease transmission via water contact or ingestion is of great concern. Sources of pollution of recreational waters include: sewage and industrial discharges, solid waste disposal, stormwater run-off, animal populations, and agricultural drainage (57). These aquatic environments can act as a vector for transmitting such fecal-oral pathogens as: *Shigella* spp., *Giardia lambia*, *Vibrio cholera*, *Entamoeba histolytica*, *Salmonella* spp., *Klebsiella* spp., enterotoxigenic *Escherichia coli*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Francisella tularensis*, *Leptospira* spp., enteric viruses, and *Cryptosporidium* spp. In the three year period from 1986 to 1988, 26 outbreaks due to recreational water use

were reported. Those outbreaks included cases of *Pseudomonas* dermatitis associated with the use of hot tubs and shigellosis, giardiasis, and viral infections from swimming at bathing beaches (29).

Constructed wetlands for domestic wastewater treatment. Inadequate treatment of domestic wastewater can contribute to non-point source pollution of surface waters and contaminate ground water supplies. The potential for contamination of surface and ground waters, especially in rural areas, may be due to the reliance on septic tank systems for household waste disposal. For example, as of 1993 in Alabama, 78 percent of the homes on septic tank systems were in rural areas with an estimated 50 percent of the total households on septic tank systems depending on ground water for drinking water supplies. In 1989, 50 percent of these private well sampled had high bacteria counts, up from 35 percent in 1973 (49). A major contributor to this serious health problem may be septic tank systems that are improperly designed and/or constructed or are at the limits of their engineered life span. An examination of the bacteriological quality of wells and springs in rural Preston County, West Virginia found that 68 percent of the supplies were in violation of the USEPA maximum contaminant level (MCL) of one total coliform per 100 ml (51,52). A re-examination of 47 of those same rural West Virginia drinking water supplies found 62 percent contaminated with one or more total coliforms (11). Two factors which contribute to the incidence and severity of contamination are: depth to the water table and distance of the ground water source from the discharge zone of the septic tank system (15). Moreover, it is not uncommon for multiple subsequent

owners of older rural homes to be unaware of the location, age, or type of septic tank system in use on their property.

Alternatives to conventional on-site wastewater treatment systems are needed to help mitigate and prevent surface- and ground-water contamination. Small constructed wetlands are believed to be an effective treatment alternative where conventional septic tank systems are rendered ineffective due to poor or shallow soils, high ground water table, karst topography, limited lot size, age of the system, lack of maintenance, or improper design and/or installation (49). In addition, they offer a potentially low-cost and low maintenance biological method of wastewater treatment. Constructed wetlands comprise a subset of created wetlands that are designed and developed specifically for water treatment. They consist of a water or effluent flow, specific aquatic macrophyte populations, waterproof liner, and a matrix which, in varying combinations, help improve the water quality (21). Wetland systems can effectively treat significant levels of BOD, suspended solids, nitrogen, metals, trace organics, and pathogens, and to a lesser degree, phosphorous (41). Constructed wetlands work because of a symbiotic relationship between the aquatic macrophytes and the microbes associated with the plants. The microbes alter contaminant substances to obtain nutrients or energy to carry out their life cycles (24).

As of 1995, there were at least 500 managed constructed wetland systems in operation in the U.S. (41). They treat effluents and wastewaters from towns, paper mills, landfill leachate, failed septic tanks, agricultural inputs, food processing plants, petroleum refineries, mine drainage, and other industrial sources ranging in elevation

from sea level to 5000 feet and in varying climates from the tropics to the sub arctic (24).

There are two types of constructed wetlands utilized in the U.S. The first is classified as a *free-water-surface* (FWS) wetland, in which the water surface is exposed to the atmosphere. A layer of soil is used as a rooting zone with emergent aquatic macrophytes functioning as a part of the treatment component of the system. The second type is called a *subsurface-flow* (SF) wetland where the water level is maintained below the surface of the porous material used as a rooting medium, usually gravel. The same types of plants are used as in the FWS wetlands. Most of the wetlands in use in the U.S are the SF type. The FWS wetlands are mainly used for treating acid mine drainage and ash pile drainage in coal producing regions (41).

There are five major components in waste water which are affected by constructed wetland systems: suspended solids, BOD, nitrogen, phosphorous, and pathogens (12). The removal or reduction of these components is mediated by the relationships between the physical, chemical, and biological components of a constructed wetlands system (Figure A). Suspended solids are removed in the wetland by sedimentation and filtration – both physical processes. These small particles may become additional attachment sites for the wetland microbes. However, clogging of pore spaces can occur which can reduce the hydraulic conductivity of the wetland matrix. Similarly, pathogens are either filtered by the wetland matrix or are retained long enough in the inhospitable wetland environment to become non-viable. Average reduction rates of indicator organisms of 1-2 logs in

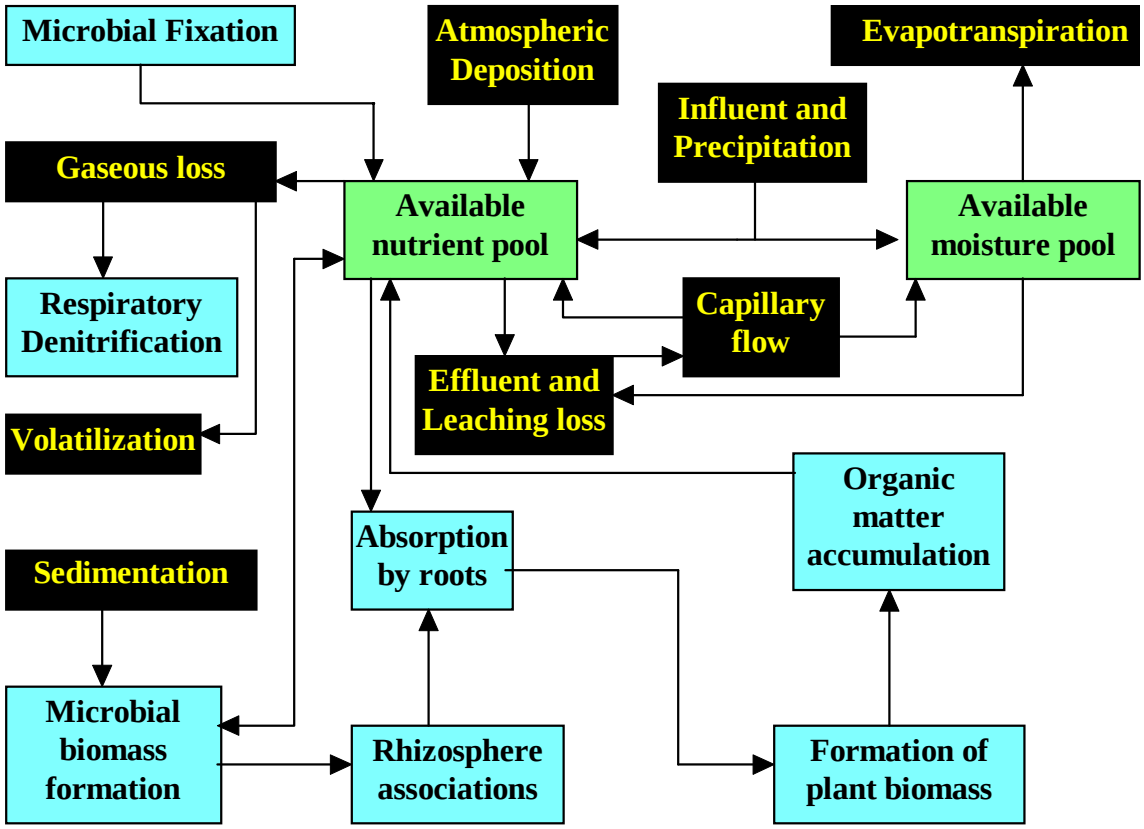

Figure A. Relationships between the physical, chemical, and biological components of a constructed wetland system.

systems with 3-7 day residence times and 3-4 logs in systems with residence times of greater than 14 day are noted in the literature (41). It has been suggested that certain root exudates from the macrophytes may have an antibiotic effect, but no direct evidence has been reported (12). These wetland systems are also effective in treating high levels of BOD. Microbes are the primary agent for removal of this soluble organic matter from wastewaters. The soluble organic compounds are mainly degraded aerobically by these microbes, although anaerobic degradation can occur (12).

Phosphorous removal in wetland systems is not very efficient because of the limited contact opportunities between the wastewater and the soil and the finite capacity of the soil to absorb the phosphorous. Systems may show very effective phosphorous removal during the first year of two of operation due to soil adsorption and vigorous early plant growth (41). Once the system reaches equilibrium, available soil sites are filled and the plants have reached the limit of their expansion. Continued plant uptake will occur, but this is offset somewhat by phosphorous release due to litter decomposition. Cutting the plants and removal of the debris will remove some phosphorous, but this is not an efficient practice, from a cost or maintenance standpoint, on a regular basis.

The removal of nitrogen is the result of complex biological transformations including immobilization, mineralization, nitrification, and respiratory denitrification. These transformations are strongly influenced by the redox status of the soil matrix in the wetland. Nitrogen enters the wetland in the form of organic N, NH_4^+, NO_2^-, and NO_3^-. Organic N undergoes mineralization and releases NH_4^+ to the water and soil.

Decomposing plant material and microbial biomass also contain organic N and are released in this manner. Nitrification followed by respiratory denitrification is believed to be the pathway of NH_4^+ removal from the wetland. Ammonia is oxidized to NO_3^- by nitrifying bacteria in aerobic zones. The NO_3^- is then converted to N_2O and N_2 by denitrifiers in anaerobic zones. Nitrification can occur in the deeper sediments of the wetland below the aerobic zone due to transport and mass flow of oxygen to plant roots. Some of this oxygen diffuses out from the roots creating a narrow aerobic rhizosphere. This transfer of oxygen to the roots is relatively slow requiring a designed residence time of about a week in warm weather. Plant uptake of nitrogen occurs, but this route removes a minor fraction of the total; estimates of around 10 percent of the total nitrogen removed have been suggested (41).

Sanitary indicator organisms and their disadvantages. To ensure safe recreational water and a continued supply of potable water, frequent monitoring of both raw water sources and finished products for the presence of pathogens is very important (48). However, methods of detection and enumeration of any or all pathogenic organisms that may be present in an aquatic sample can be complicated and time consuming, often does not give the relative quality of a water supply, and for some organisms, do not yet exist. In addition, there is no single procedure for detecting all waterborne pathogens and there is concern for laboratory workers handling pathogenic organisms (42,43). Instead, the most effective way to monitor microbiological water quality is through simple, rapid, and relatively inexpensive tests for fecal indicator organisms.

Microbial examination of drinking and recreational waters, via bacterial indicator organisms, has proven useful in protecting the public health and preventing the spread of waterborne disease. The ideal indicator of fecal pollution should be present simultaneously with the pathogen(s), be specific for fecal contamination, be able to resist water treatment and disinfection processes to the same or a slightly greater extent than the pathogens, have no aftergrowth in external aquatic environments, have similar survival characteristics to the pathogen in the environment, and be detectable by simple and rapid methods (28,48). It is also desirable to differentiate the source (i.e., man versus animal) of the pollution (28). Presently, total coliforms, fecal coliforms, and enterococci are examples of just a few of several indicators in use, none of which meet all of the aforementioned criteria. However, these indicators are considered to be the classical microbiological parameters of water quality and have proven useful for indicating the presence of pathogens of fecal origin (56).

Several indicator organisms have been used to monitor the bacteriological quality of water. Bacteria such as total coliforms, fecal coliforms, fecal streptococci, *Clostridium perfringens*, and heterotrophic plate count bacteria (HPC) have been used. Even viruses such as bacteriophages, especially coliphage, have been suggested as potential indicators. However, each group listed is a less than ideal indicator.

Total coliform (TC) bacteria, used for nearly 100 years in the U.S., is employed today in many countries to monitor fecal contamination and indicate the microbial quality of raw and finished water (35). Overall, these indicator organisms apparently continue to ensure, with a few exceptions, that drinking water shall be safe

for human consumption (56). TC are described as gram-negative, aerobic, or facultatively anaerobic, non-spore forming rods that ferment lactose producing gas in 48 hours at 35°C. This group of organisms includes the genera *Escherichia*, *Enterobacter*, *Citrobacter*, and *Klebsiella*. They are part of the lower intestinal tract of man, usually excreted in about 10^{11} organisms per person per day (57). Since enteric pathogens are excreted simultaneous with this group of organisms, the presence of TC indicates possible fecal contamination and the potential presence of enteric pathogens.

The 1986 amendments to the Safe Drinking Water Act (SDWA) changed how the USEPA regulated the use of TC in monitoring drinking water. Before these amendments, TC were monitored based on their density using the multiple tube fermentation (MTF) or the membrane filtration (MF) technique. This requirement changed when the EPA proposed a new rule on November 3, 1987 that would allow the monitoring of TC based on a presence-absence (PA) method. This decision was based on studies that demonstrated that the level of coliform bacteria was not quantitatively related to the potential for an outbreak of waterborne disease, while the PA method provided adequate water quality information (4). The final rule was published June 29, 1989 and took effect on December 31, 1990. Analysis of TC may now be conducted using the MF, 10 tube MTF, PA, or the minimal media ortho-nitrophenyl-β-D-galactopyroside-4-methylumbelliferyl-β-D-glucuronide (ONPG-MUG) test, currently commercially available as Colilert® (38).

Although TC may sufficiently meet most of the indicator criteria when used to test finished drinking water, there are several serious problems with the general use of

TC as indicators, especially when monitoring the quality of drinking water supplies in tropical climates and untreated ground water supplies (14,17,56). TC, especially the genus *Klebsiella*, exhibit aftergrowth in water of low organic matter content as well as in low temperature water (18,57). The presence of excessive background bacteria, a situation found in high organic load tropical waters and sometimes ground water but not encountered with treated supplies, can interfere with the enumeration of TC, which could possibly lead to an overestimation of drinking water quality (51,52). Many organisms in this group have no sanitary significance. They have been recovered in soil, on vegetation, and in forest and farm products, some of which were untouched by humans. Also, it has been noted that some pathogens appear to be more hardy than the TC in the environment, with a few documented cases in which pathogenic organisms were isolated from water that was negative for coliforms (56).

Fecal coliforms (FC), also called "thermo-tolerant" coliforms, are a subset of the total coliform group. In addition to exhibiting the characteristics of the TC, the FC are also able to ferment lactose with the production of acid and gas in 24 hours at 44.5°C. This group of organisms includes *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., and *Escherichia* spp. Since most FC have been shown to die at a faster rate than non-FC in an aquatic environment, presence of FC indicate recent contamination (4). There are three ways to test for FC in an aquatic environment: MPN, MF, or ONPG-MUG (2).

FC have proven to be valuable indicators of fecal pollution in many situations where TC are ineffective due to the widespread occurrence of TC in the environment (56). In temperate climates, specific enumeration of *E. coli* is effective in

demonstrating fecal pollution of water. However, as Carrillo et al. (14) demonstrate, *E. coli* was able to survive, remain physiologically active, and regrow at rates that were dependent on nutrient levels of the ambient waters in a tropical ecosystem. In addition, Carrillo et al. (14) state that less than 30 percent of the fecal coliform-positive isolates from a variety of sites around Puerto Rico are identified as *E. coli*. These tropical forest watersheds have been known to have high fecal coliform counts in the absence of any identifiable fecal source (14). Also, the FC tests are complicated in the tropics by the presence of 44.5°C positive *Citrobacter* and *Klebsiella* strains (17).

Fecal Streptococci (FS) are the third most commonly employed indicator organisms for establishing the presence of sewage in a water supply. FS usually are present in the lower intestinal tract of both warm blooded animals and man. These organisms are gram-positive cocci and include members of the genera *Streptococcus* and *Enterococcus.* The enterococci subgroup originates mainly from human fecal material and is, therefore, a better indicator of human fecal contamination than the streptococcus group. Analysis of the enterococci only, however, may not provide a good indication of the total water quality since fecal contamination from other warm-blooded sources would be missed (57). These organisms rarely multiply in polluted water, with the exception of some naturally occurring *Enterococcus faecalis* biotypes. However, they have comparable survival to both TC and FC outside the animal host (56).

In animals, the FS usually outnumber the FC, while in man they are much fewer in number (57). FS serve as an index of fecal pollution of raw water and may

provide additional information on the source of the contamination when used in a ratio with the FC counts. Because man harbors a greater population of FC compared to FS, whereas the reverse is true for other warm-blooded animals, a FC:FS ratio has been employed to help identify the source of the pollution. A ratio greater than 4.0 usually identifies man as the source of contamination, while a ratio of less than 0.7 indicates contamination of animal origin.

Like the TC and FC, the FS can be used as fecal pollution indicators in water. Although these organisms occur naturally in the environment, their presence in high numbers, especially if FC are also present, indicate contamination has occurred. Because of these naturally occurring species, it is very important to include FC and FS density information to determine whether fecal contamination has actually occurred. For this reason, it is not recommended that the FS be used as the sole indicator of fecal pollution (56).

Bifidobacteria. The genus *Bifidobacterium* is characterized as strictly anaerobic, nonspore-forming, nonmotile, gram-positive, thick pleomorphic rods that inhabit the gut of animals and humans. They may exhibit branching bulbs, clubs, coryneforms, buds, spheroids, and bifurcated Y and V forms when freshly isolated from fecal sources. Their morphology is widely affected by nutritional conditions and, hence, will appear quite different when isolated from an aquatic environment as opposed to fresh feces (10,28). Culturing these organisms on normal laboratory media, such as MRS broth or reinforced clostridial media, greatly reduces pleomorphism (56). Bifidobacteria are catalase negative and produce acetic and lactic acids without gas during glucose fermentation.

Bifidobacteria are major components of the intestinal flora of human beings throughout their life cycle. There is evidence that bifidobacteria are required to maintain the proper balance of human intestinal microflora for adequate digestion (46). They are considered to be beneficial for humans at all age groups and are thought to have anticarcinogenic and anticholestolemic properties (27). These organisms play a significant role in controlling the pH of the large intestine through the production of acetic and lactic acids which restricts the growth of many potential pathogens and putrefactive bacteria. In fact, several growth factors produced by bifidobacteria are used in different combinations to modify cow's milk to help promote growth of bifidobacteria in nursing infants. In adults with antibiotic-induced diarrheas, administration of a bifidobacterial inoculum, usually through yogurt or milk, will correct this problem (37). Pharmaceutical companies have also added bifidobacteria to some of their products which aid in the treatment of enterocolitis, constipation, liver cirrhosis, and for the promotion of intestinal peristalsis (10).

Bifidobacteria were first described by Tissier in 1900 (9,28), who discovered them in the feces of infants and named them *Bacillus bifidus communis*. Although the genus *Bifidobacterium* was recognized as a separate taxon in 1924, the seventh edition of *Bergey's Manual of Determinative Bacteriology* referred to these organisms collectively as *Lactobacillus bifidus*. In the latest edition of *The Prokaryotes* (10), 24 species of the genus are listed.

Bifidobacteria and the fructose-6-phosphate shunt (F6P shunt). Perhaps the most direct and reliable method for classifying a bacterial strain as belonging to the genus *Bifidobacterium* is the one which demonstrates the presence of the enzyme

fructose-6-phosphate phosphoketolase (F6PP) in cellular extracts of the bacteria (9,10). F6PP is the key enzyme of bifidobacterial hexose metabolism and its presence is heretofore considered unique to the genus. The hexose fermentation pathway in bifidobacteria has been elucidated (10). In this pathway, F6PP splits fructose-6-phosphate to erythose-4-phosphate and acetyl phosphate. From there, through the successive action of transaldolase and transketolase, pentose phosphates are formed which give rise to lactic and acetic acids in a 2:3 ratio (see Figure B). It is also interesting to note that this pathway produces 2.5 mol of ATP per mol of glucose compared to the 2.0 mol ATP per mol of glucose in the homofermentative pathway and 1.0 mol ATP per mol of glucose in the heterofermentative pathway (22).

Munoa and Pares in 1988 (35) reported a *Bifidobacterium* isolation medium which took advantage of this shunt through the incorporation of iodoacetic acid (IA) in the medium. IA inhibits a key enzyme in glycolysis, glyceraldehyde-3-phosphate dehydrogenase (see Figure C). This inhibition "favors" hexose fermentation via the F6P shunt and, as reported by Munoa et al. (35), drastically reduced the number of non-bifidobacterial contaminant colonies on the plating medium.

Bifidobacterium spp. as indicators. As indicated previously, bifidobacteria possess the following advantageous properties as indicators: 1) they are exclusively of fecal origin; 2) they exist in high densities to allow detection; 3) some species are present only in the human intestinal tract; 4) they are obligate anaerobes and do not

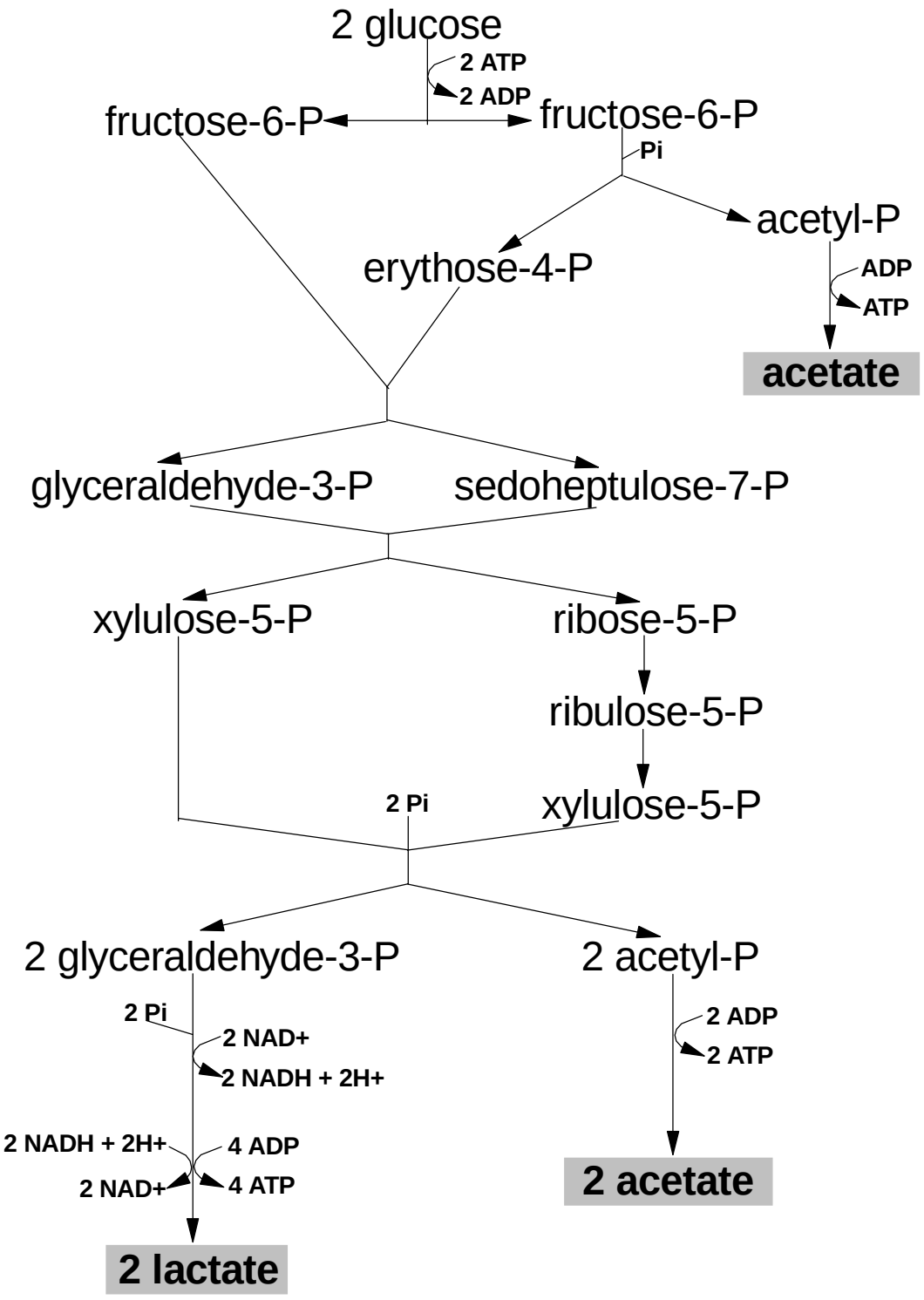

Figure B. The hexose fermentation pathway of the genus *Bifidobacterium* (Gottschalk).

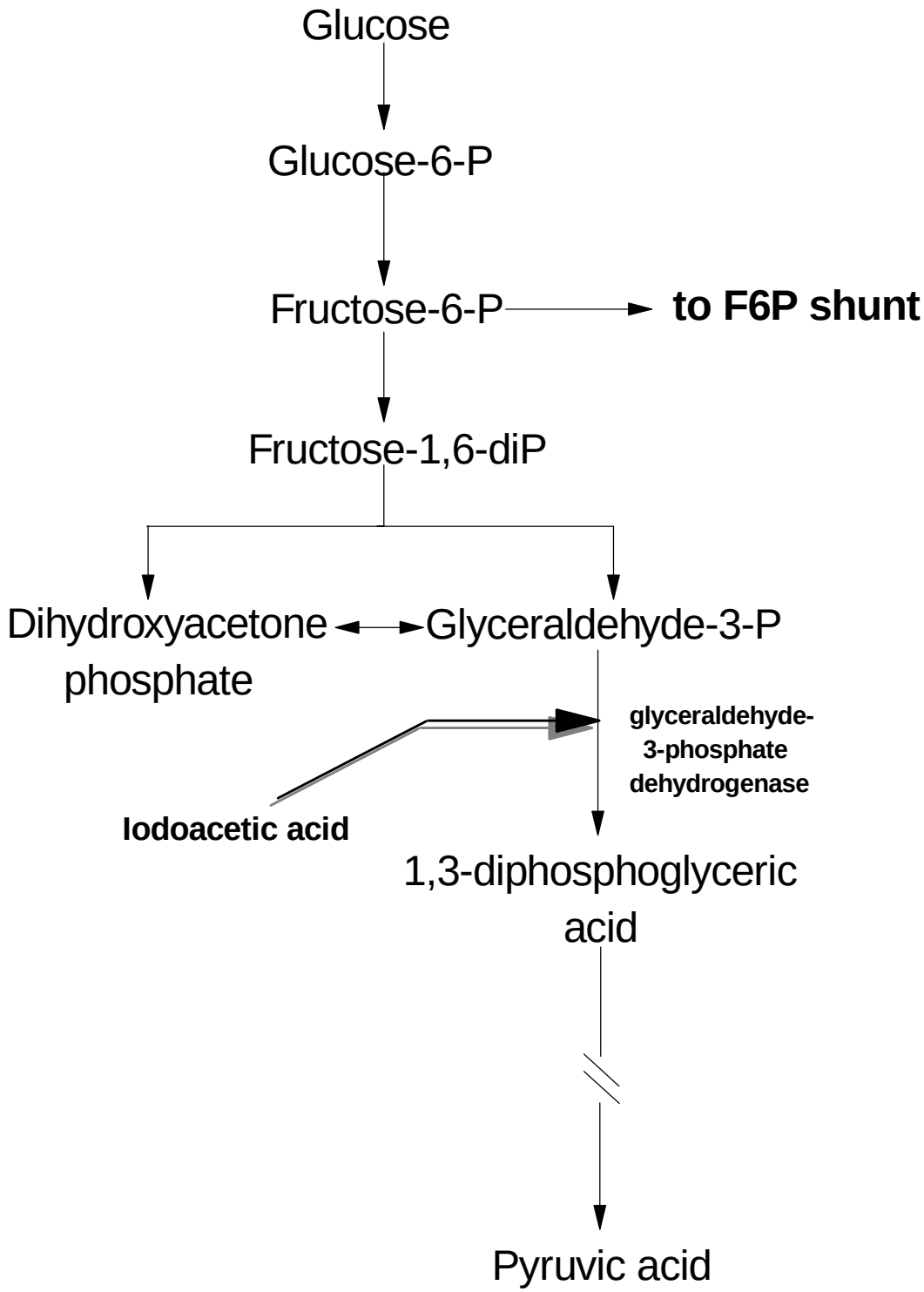

Figure C. Glycolysis showing fructose-6-phosphate shunt and inhibition of the pathway by iodoacetic acid

grow well in aquatic environments; and 5) they have similar survival characteristics to *E. coli* in surface waters (43). Five species, *B. adolescentis, B. bifidum, B. breve, B. infantis,* and *B. longum,* are found exclusively in humans. These species are found in the gut of all humans within 6 days of birth (14). Bifidobacteria are the predominant component of human intestinal microflora, typically reaching densities of 10^9 to 10^{11} cells per gram of feces (9,28). Significant to drinking water quality monitoring, enumeration of these species from environmental samples may permit reliable differentiation between animal and human sources of pollution (28).

Despite being first described in 1900 by Tissier (28), there is limited ecological information on the genus. The potential significance of bifidobacteria as indicator organisms was first proposed by D.A.A. Mossel in 1958 (34). However, even with the aforementioned advantages, there is a deficiency of available data on the behavior of *Bifidobacterium* spp. in external aquatic environments, particularly in ground water (28). Studies of bifidobacteria in fecal specimens from humans have shown that the population is higher and more constant than *E. coli* or fecal streptococci (enterococci) across all age and ethnic group boundaries (18,23)

Several authors have investigated the potential significance of using bifidobacteria as fecal pollution indicators in water (14,17,18,23,31,42,43). Most of these studies have concentrated on using raw sewage samples (17,18,31,43) or grossly contaminated surface waters (14,35). Studies with pure cultures of *B. bifidum*, *B. adolescentis*, and *B. pseudolongum* have demonstrated clearly that bifidobacteria are unlikely to multiply in raw waters because: very little growth occurs above or below the extremely narrow temperature range of 30 to 40°C; no growth can occur above 7

percent atmospheric oxygen; and maximum growth rates can occur only in the presence of high concentrations of proteins or carbohydrates (56).

As early as 1960, Gyllenberg et al. (23), in evaluating the suitability of the bifidobacteria as indicators of fecal pollution in water, were conducting comparison studies of the bifidobacteria, coliforms, and enterococci in water under various conditions. In their experiments, feces of healthy adults were collected, mixed, suspended, and diluted in well and lake waters. The bifidobacteria, coliforms, and enterococci were then enumerated over time. Their data showed no difference in survival of the indicator organisms in the different waters or when using different concentrations of feces. However, differences in indicator occurrence and survival were noted when temperature was used as the variable. Initial counts of coliforms and enterococci were approximately 10^6 to 10^7 per gram of feces, whereas corresponding figures for the bifidobacteria were considerably higher with counts in the 10^8 to 10^9 per gram of feces. After two days of storage at room temperature, numbers of bifidobacteria fell while the number of coliforms and enterococci increased by 208 and 144 percent, respectively. From the second day on, numbers for all three groups of bacteria decreased. The reduction rate of the coliforms and the bifidobacteria were of the same order of magnitude and the rate for the enterococci was somewhat lower. At refrigerator temperatures, the numbers of all three groups of indicators were slightly reduced. Again, the coliforms and the bifidobacteria had almost identical reduction rates whereas the enterococci were reduced at a lower rate. Gyllenberg et al. (23) concluded that bifidobacteria survive similar to coliforms under the conditions found in natural waters. The main difference in the behavior of the

indicators was that the bifidobacteria were definitely incapable of multiplication under these conditions whereas coliforms may show an initial increase.

In 1975, Evison and James (17) investigated the distribution of bifidobacteria, coliforms, *E. coli*, and enterococci in a variety of water and other samples in the United Kingdom (U.K.) and Africa. They also compared the response of bifidobacteria to temperature and organic content of the water to that of the other indicators in field and laboratory studies. Water samples were collected in the U.K., Kenya, and Morocco. They found that, at warmer temperatures (26 to 30°C), the death of enterococci was very rapid, even if the sample was supplemented with glucose, thus detracting from its value as an indicator in tropical conditions. Under similar conditions, coliforms and *E. coli* exhibited aftergrowth, as much as one thousand-fold in some cases. Bifidobacteria were less affected by the organic content of the water although slight regrowth was shown to occur when the sample was supplemented with 200 mg/l of glucose. Evison and James (17) concluded that their preliminary study showed that bifidobacteria have most of the characteristics required of a bacterial indicator of pollution, and in tropical situations, they may behave more consistently than other accepted indicator species. As a result, "further examination of this group as a fecal indicator is justified and necessary" (17).

Carrillo et al. (14) examined the distribution, density, and *in situ* survival of *E. coli* and bifidobacteria in a tropical rain forest watershed in Puerto Rico. Water samples were taken from six sites along the Mameyes River in northeastern Puerto Rico. They found that the highest densities of fecal coliforms (FC), *E. coli*, and bifidobacteria were recorded at a site below a sewage outfall. Densities of FC and *E.*

coli were two orders of magnitude higher than at the other sites; however, densities of FC and *E. coli* at all sites exceeded recommended coliform MCL for potable waters. Bifidobacteria did not show large increases at sites known to contain sewage contamination. Unlike the work reported by Gyllenberg et al. (23) and Evison et al. (17), which showed that densities of bifidobacteria were always greater than densities of *E. coli* or FC in contaminated waters, all sites in the rain forest watershed had higher densities of bifidobacteria than *E. coli* and FC except the site below the sewage outfall. Carrillo et al. (14) also demonstrated that *E. coli* could survive indefinitely in the rain forest watershed and exhibited aftergrowth. In contrast, bifidobacteria decreased significantly over time, declining more than 50 percent in 48 hours. Carrillo et al. (14) concluded that the primary regulator of densities of indicator bacteria in the tropical environment was the nutrient concentration of the water. They suggest that coliforms not only survive but become part of the normal flora in tropical freshwater environments. Bifidobacteria, on the other hand, show promise as an indicator of recent fecal contamination in terms of lack of survival *in situ* and specifically as a human fecal indicator (14).

Bifidobacteria enumeration media. One problem which has surfaced in recent studies is the lack of a reliable selective medium for the isolation and enumeration of *Bifidobacterium* spp. (46). Results obtained when raw water was examined for bifidobacteria differ according to the isolation medium used. Another major disadvantage with these media has been the inability to demonstrate reproducibility and reliability in the enumeration of bifidobacteria. Also, these media tend not to be as selective when used with surface water samples as they are with sewage samples

(56). As a result of an absence of a reliable selective isolation medium, there is a lack of available information concerning the extraenteric behavior of bifidobacteria (35).

Many selective agents have been used to better enumerate bifidobacteria. Antibiotics, such as kanamycin, neomycin, paromycin, nalidixic acid, and polymyxin, have been incorporated into both synthetic medium formulations and manufactured complex substrates, e.g. reinforced clostridial medium (Difco). Other compounds, such as sodium propionate, lithium chloride, sorbic acid, sodium azide, bromocresol green, and iodoacetic acid, have been utilized as selective and differential agents. In some cases, the use of these selective media can be useful (i.e., sewage and fecal samples), but due to great intraspecific variation in the resistance of bifidobacteria to antibiotics, none of the selective media proposed thus far seem to guarantee complete selectivity, nor do they allow for the growth of all the species of bifidobacteria (9). This problem is further compounded when aquatic samples which are suspected of fecal contamination are processed using a bifidobacterial selective medium; this is especially true of temperate surface waters. Because of their rather complex growth and maintenance requirements, bifidobacteria quickly become injured in these aquatic environments, and hence they tend to be noncultivatable without employing resuscitative techniques (35).

Bifidobacteria and molecular biology. *Bifidobacterium* spp. have long been differentiated based on their fermentative characteristics and cellular morphology, but this often led to taxonomic uncertainties (10,28,31,33,39). In 1970, Biavati et al. (10) started to extensively apply the DNA-DNA filter hybridization procedure in order to assess the validity of the bifidobacterial species previously described. By 1982, the

use of polyacrylamide gel electrophoresis of soluble cellular proteins was employed to give a better assessment of the genetic relationships among the different species (10,45).

Yamamoto et al. (59) used 16S rRNA species-specific oligonucleotide probes to detect the five human indigenous bifidobacteria. Those five probes were highly species specific for strains of the human intestinal tract. They concluded that this RNA-DNA hybridization technique is more rapid, simple, and sensitive than methods based on phenotypic characteristics or DNA-DNA homology.

Mangin et al. (30) were able to differentiate between strains of the same species of bifidobacteria by using rRNA restriction patterns. Total DNA from 21 collection or industrial strains of bifidobacteria were cleaved with BamHI, EcoRV, and PvuII restriction endonucleases. The resulting bands were subjected to Southern blot hybridization with a labeled rDNA 23S gene probe. The patterns allowed all tested strains to be differentiated and for the collected strains, previous classifications to be confirmed.

More recently, Bernhard and Field (8) were able to utilize *Bifidobacterium*-specific 16S ribosomal DNA primers to identify nonpoint sources of fecal pollution in coastal waters. They utilized the polymerase chain reaction with genus specific primers coupled with terminal restriction fragment length polymorphism (T-RFLP) analysis to identify these organisms in water samples frequently contaminated with fecal pollution.

Summary. Increasing utilization and contamination of freshwater supplies, especially ground water, is a cause for public concern. This problem, which has been

occurring for centuries, is worsening due to use of increased industrial, agricultural, and municipal activities. This contamination has resulted in many cases of waterborne disease in the U.S. (20,29). People who rely on ground water as their source of drinking water are especially vulnerable to this health hazard. In rural areas, inadequate treatment of wastewater further contributes to the deterioration of ground water quality. Alternative systems such as constructed wetlands, show promise as an effective treatment of domestic wastewater when used in lieu of a leachate field in a private septic system.

The examination of water samples for fecal indicator organisms has proven to be effective in ensuring safe water supplies and protecting the public health. Organisms such as total coliforms, fecal coliforms, and enterococci are examples of indicators currently in use, but they are generally considered less than ideal for detecting the presence of pathogens in all water supplies.

Bifidobacteria, of all the indicator systems thus far proposed, appear to be the most specific for fecal contamination and the one potentially most able to reliably distinguish between human and animal wastes. Their characteristics include:

- Bifidobacteria are exclusively of fecal origin. There are no known extraenteric sources of these organisms, beyond those already fecally contaminated
- Unlike other indicator systems, they do not multiply in aquatic environments.
- They exist and are excreted in densities surpassing those of other indicators, including *E. coli*.
- There are five species specifically indigenous to humans which would allow the differentiation of the source of contamination.

Current disadvantages of employing bifidobacteria include: the difficulty in working with anaerobes; lack of an acceptable enumeration medium; and deficiency of data on their behavior in external aquatic environments.

OBJECTIVES and JUSTIFICATION

The objective for this project at the outset was to test the feasibility of using bifidobacteria as an adjunct to the coliform test to help identify the incidence and the source of fecal contamination in water. The original intent was not to propose replacing current accepted indicators, but to offer a supplemental test that could be conducted concurrently. Problems at the outset of the project included a lack of information on the behavior of bifidobacteria in the various aquatic environments in which they would eventually have to be tested. Additionally, what information there was, many times, tended to conflict. For example, Evison and James (17) found that survival of bifidobacteria was equal to and sometimes greater than the survival of *E. coli* in laboratory studies. Resnick and Levin (42,43) suggested that survival of bifidobacteria is poor in the aquatic environment while coliforms survived better. Another major problem with using this group of organisms has been the lack of a reliable selective medium for the enumeration of bifidobacteria in the environment. It has been suggested that the high concentrations of selective agents in the various bifidobacteria selective media found in the literature may interfere with the recovery of injured bifidobacteria (56). Therefore, it was decided at the beginning of the project to better characterize the behavior of bifidobacteria in a controlled aquatic environment; both their survival and injury attributes on selective and non-selective media were examined. An attempt was made to simulate both aerobic and anaerobic circumstances that would resemble surface and ground water conditions. It was

hoped that by completing these microcosm studies that a better understanding of those parameters would help optimize the efficiency of recovery of bifidobacteria from an aquatic sample. The information gleaned from the microcosm studies was then applied to studying the occurrence of bifidobacteria in constructed wetlands receiving primary treated sewage. In these samples, bifidobacteria were enumerated, isolated, and differentiated based on their carbohydrate fermentation characteristics.

CHAPTER 1

Evaluation of Survival and Recovery Characteristics of Bifidobacteria in Microcosm Experiments

INTRODUCTION

Examination of aquatic environments for bacterial indicator organisms has proven to be a useful and well-established practice for the purpose of monitoring microbiological water quality. Instead of using complicated and time-consuming methods to monitor pathogens, specific tests for enteric indicator bacteria have been developed. An ideal indicator of fecal pollution should be: 1) present in sufficient density to allow detection, 2) present simultaneously with pathogen(s), 3) incapable of aftergrowth in external aquatic environments, 4) easy to enumerate, and 5) exclusively of fecal origin (28,48). Unfortunately, none of the indicator organisms presently in use today meet all of these requirements.

Bifidobacteria show promise as indicators of fecal pollution in water. They are exclusively of fecal origin, exist in high densities in feces, are obligate anaerobes with complex nutrient requirements, and have the potential to distinguish between fecal pollution of human and animal origin (43). However, even with these advantages, there is a lack of information concerning the behavior of bifidobacteria in aquatic environments. This is due, in part, to a lack of an acceptable selective isolation medium as well as the difficulty involved in working with anaerobic bacteria.

In order to better characterize the behavior of bifidobacteria in a controlled aquatic environment, survival and injury parameters on selective and non-selective media were examined. Findings from these *in vitro* experiments should provide a

better understanding of those parameters that contribute to the recovery efficiency of bifidobacteria from aquatic environments.

MATERIALS AND METHODS

1.2.1 Preparation of cells for storage. One hundred ml of Reinforced Clostridial Medium (RCM, DIFCO, Detroit, MI) was inoculated with *Bifidobacterium bifidum* ATCC 29521 and incubated anaerobically at 35ºC for 96 h. Following incubation, cells were centrifuged at 6000 X *g* for 20 min at 4ºC. The supernatant was decanted. The pellet was resuspended in 100 ml of a 10 % glycerol/water solution, and then centrifuged under the same conditions. These steps were repeated resuspending cells in 10 %, 15 %, and 20 % glycerol solutions in successive repetitions. The pellet was finally resuspended in 5.0 ml of a 20 % glycerol solution and apportioned into 0.5 ml aliquots in 1.5 ml Eppendorf tubes. The tubes were stored at -80ºC until used. (Note: A limited number of experiments were conducted with *B. longum* ATCC 19755. Preparation of cells of this strain was identical to that described for *B. bifidum*. Observations for *B. longum* can be found in the Appendix.)

1.2.2 Growth curve determinations. Five modified 300 ml side-arm flasks (Figure 1.2.1), containing 100 ml RCM and 0.1 ml of a rezazurin stock solution (0.5 g/L) were used for growth curve determinations. Three of the flasks were inoculated with 0.1 ml of *B. bifidum* and two flasks remained uninoculated. Of the two uninoculated flasks, one was a sterile anaerobic control and the other was used as an oxygen exposed control.

Before inoculation, all flasks were assembled as shown in Figure 1.2.1, except for the addition of the electrical tape. The flasks were autoclaved and then allowed

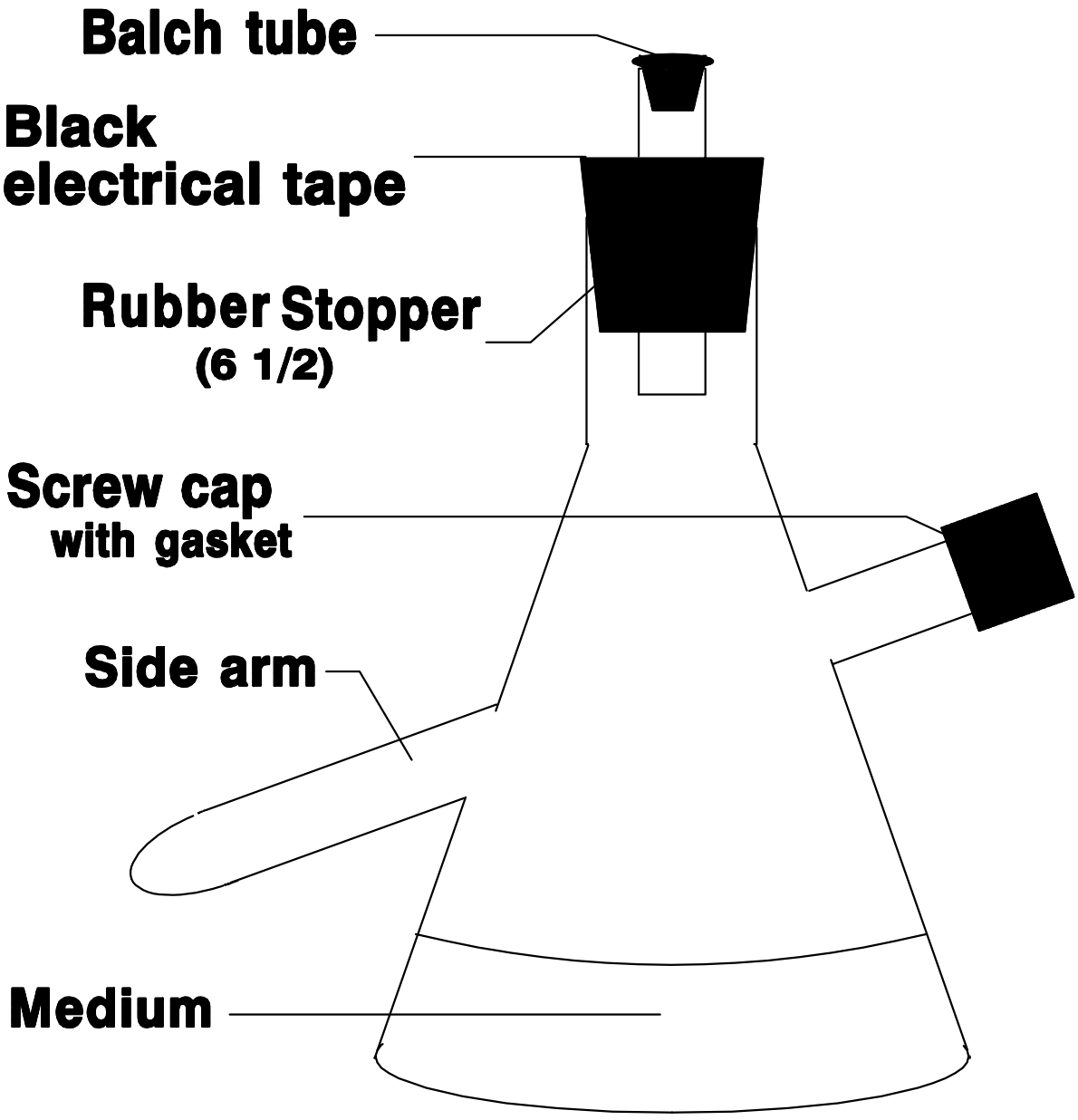

Figure 1.2.1. Side arm flask. Notes: Balch stopper made from neoprene; custom fitted rubber gasket inserted very tightly inside screw cap.

to cool under CO_2 injected through the Balch tube. When the flasks were cool enoughto safely touch, the electrical tape was applied around the necks of the flasks and stoppers forming a tight seal. The flasks were then allowed to cool to room temperature before tightening the screw cap and overpressurizing (around 2 psi). At this point, the screw cap of one of the uninoculated flasks was loosened so that it became exposed to oxygen.

After inoculation, all of the flasks were incubated at 37°C. Growth was monitored spectrophotometrically (640 nm) using a Bauch & Lomb Spectronic 20. Each flask was swirled and then tilted until the culture filled the side arm for placement in the spectrophotometer. Periodically, a sample was withdrawn from the flask via the side arm, appropriately diluted, spread plated on Reinforced Clostridial Agar (RCA, DIFCO), and incubated at 35°C under anaerobic conditions in GasPak jars (BBL Microbiology Systems, Cockeysville, MD). During sampling, the flask was sparged with CO_2, resealed, and overpressurized again. The growth curve (plotted as absorbance and cell counts vs. time) is given in Figure 1.5.1 of the Appendix. (Note: Growth curve determination for *B. longum* is also given in the Appendix, Figure 1.5.2.)

1.2.3 Aerobic pure culture survival experiments. *Bifidobacterium bifidum* ATCC 29521 was grown from a frozen culture in RCM and incubated anaerobically at 35ºC for 96 h. One ml or 0.1 ml of the culture was used to separately seed 250 ml sponge stoppered flasks containing 100 ml of sterilized groundwater or phosphate buffer (1.25 ml phosphate buffer stock solution, 34.0 g/l; 5.0 ml $MgCl_2$ stock

solution, 81.1 g/l; 1 l distilled H_2O) in order to give an initial microcosm inoculum of 10^4 - 10^5 cells/ ml. Microcosm experiments were conducted at selected temperatures to determine the optimal selective enumeration medium and survival characteristics of bifidobacteria. All microcosms were previously sterilized by autoclaving at 120ºC for 15 min. These microcosms were stored at 13, 28, or 35°C during the course of the experiments.

Samples were removed from the respective microcosms at various times and spread-plated in triplicate onto petri dishes containing RCA. All plates for the enumeration of *Bifidobacterium* spp. were incubated at 35ºC under anaerobic conditions in GasPak jars. After incubation, colonies were counted and the replicates averaged. (Note: A few selected experiments were conducted using anaerobic microcosms. The reader is referred to the Appendix for data presentations for these experiments.)

1.2.4 Selective media evaluation experiments. In order to evaluate the efficiency of various selective media, part of the aerobic pure culture experiments were repeated under the conditions described above and the cells were enumerated on the selective media in addition to RCA. Enumeration media evaluated were: *Bifidobacterium* iodoacetate medium 25 (B25) and 50 (B50) as described by Muñoa and Pares (35) (Table 1.1); and, YN-6 medium as described by Resnick and Levin (42) (Table 1.2). B25 and B50 were used in conjunction with the 5 h resuscitation technique described by Muñoa and Pares (35). This technique involved spread-plating the sample onto a non-selective RCA bottom layer, anaerobically incubating the plates at 35°C for 5 h, and then pouring the tempered selective agar over the bottom

Table 1.1. *Bifidobacterium* Iodoacetate Medium

Ingredient	Amount[a]
Reinforced Clostridial Medium (RCA)	51
Nalidixic Acid	0.02
Polymyxin B sulfate	0.0085
Kanamycin sulfate	0.05
Iodoacetic acid (Na+ salt)	0.025[b]
2,3,5-triphenyltetrazolium chloride	0.025

[a] All amounts are grams per liter.
[b] BIM50 formulation doubles the concentration of iodoacetic acid

Table 1.2. YN-6 Medium

Ingredient	Amount[a]
Yeast extract	20
Peptone	10
Casamino Acid (vitamin-free)	8.0
Sodium chloride	3.2
Bromocresol Green	0.3
L-cysteine hydrochloride	0.4
Nalidixic Acid	0.08
Agar	15
Neomycin Sulfate stock (2.5mg/ml)	1 ml

[a] Unless otherwise indicated all amounts are grams per liter.

layer. The agar was allowed to harden and then the plates incubated anaerobically at 35°C in GasPak jars. RCA was used to determine 100% recovery and help characterize sensitivity and injury properties of the selective media. After incubation, colonies were counted, the replicates averaged, and the enumeration efficiency determined for each medium.

1.2.5 Aerobic mixed culture survival experiments. One ml of a 96-h *B. bifidum* culture grown anaerobically in RCM and 1.0 ml of a 24 h *Escherichia coli* ATCC 23559 culture grown aerobically in Tryptic Soy Broth (TSB, DIFCO) was inoculated into 100 ml of sterile phosphate buffer in sponge stoppered 250-ml-wide-mouth-flasks. These flasks were stored at 13, 28, and 35°C. Samples were removed at various times, appropriately diluted, and spread plated in triplicate onto B25, YN6, and mFC agar. All plates except the mFC plates were incubated anaerobically at 35°C. All mFC plates were incubated aerobically at 44.5°C. MFC agar was used to selectively enumerate *E. coli*, and B25 and YN6 were used to selectively enumerate the bifidobacteria.

1.2.6 Calculations and statistical analysis. The percent decrease of the cell population at some time, *x*, as a result of exposure to sterile groundwater or phosphate buffer was calculated as follows:

$$\% \text{ decrease} = \left[1 - \frac{(\text{cell population at time } x)}{(\text{cell population at time zero})}\right] \times 100$$

The decimal reduction time (DRT) or the time in which the population decreases by a 1 log was calculated as follows:

$$DRT = X_1 - X_2$$

given the equation for a straight line is: $y = mx + b$

and where:

$$X_1 = [(LOG (Y_1) - b)] / m$$

$$X_2 = [(LOG (Y_2) - b)] / m$$

b = intercept of mean regression line
m = slope of mean regression line
Y_1 = estimate of population
$Y_2 = Y_1 / 10$
X_1 = initial time
X_2 = time at 1 log Y_1 reduction

The slope and intercept of each individual replicate were used in the statistical comparisons of decimal reduction times between incubation media; however the average population estimates were used in each replicate calculation for each medium.

The percentage of the cell population injured by exposure to sterile groundwater or phosphate buffer was calculated as follows:

$$\% \text{ Injury} = \left[\frac{(\text{CFU on nonselective medium}) - (\text{CFU on selective medium})}{(\text{CFU on nonselective medium})}\right] \times 100$$

Injury induction time (IIT), showing the ability of the selective media in enumerating the bifidobacterial population, was calculated from determining the slope of the regression line through a log difference versus time plot for each selective medium.

Log difference = LOG (population on RCA at time x) – LOG (population on selective medium at time x)

The slope and intercept of this regression line was then inserted into the following equation:

$$IIT = (A - m)/b$$

where:

A = number of log injury (e.g., 90% injury = 1, 99% = 2, etc.)

m = slope of the regression line

b = intercept of the regression line

Statistical procedures were computed using Excel 7.0 (Microsoft Corporation) and SigmaPlot and SigmaStat (Jandel Scientific Software, San Rafael, CA). Data for bacterial recovery from microcosm experiments were transformed to $\log_{10}$ values prior to statistical analysis.

RESULTS

1.3.1 Survival and recovery characteristics of bifidobacteria in pure and mixed culture microcosms. Phosphate buffered water and sterilized groundwater microcosms were used to simulate conditions that would be encountered by bifidobacteria in external aquatic environments. Aerobic pure culture microcosm studies were completed to evaluate recovery of bifidobacteria on selective and nonselective media at three incubation temperatures. Starvation induced injury and recovery efficiency of the selective media were documented in these studies. Aerobic mixed culture microcosms studies were completed to evaluate the survival characteristics of bifidobacteria compared to *E. coli* on selective media at three incubation temperatures. (Note: Limited anaerobic pure and mixed microcosm studies were completed with *B. bifidum*. Data presentations of these results can be found in the Appendix.)

Bifidobacteria were sampled from the aerobic microcosms and enumerated on specific selective media to investigate their injury characteristics on the published selective media. Injured bifidobacteria were shown to occur in these experiments when enumerating the cells on accepted bifidobacteria enumeration media. RCA was used as a non-selective medium and compared with the bifidobacteria selective media B25, B50, and YN6. The proportion of cells enumerated only on RCA, but not the selective media from the same sample, were considered injured.

1.3.2 Aerobic pure culture microcosms. Initial experiments involved determining the survival characteristics of *B. bifidum* in phosphate buffered water compared to groundwater. The system that produced the poorest survival rates would then be used in the remaining microcosm experiments.

Survival of *B. bifidum* at both 35 and 13°C was greater in sterilized groundwater than in phosphate buffer (Figures 1.3.1 and 1.3.3) ($P = 0.05$ and 0.10, respectively). Populations of *B. bifidum* at 35°C declined rapidly in both microcosms becoming non-detectable before 25 h incubation in phosphate buffer and decreasing approximately 99% in groundwater. Decimal reduction times in groundwater were significantly longer than in phosphate buffer ($P = 0.05$) (Table 1.3). No difference in the survival of *B. bifidum* was found at 28°C in phosphate buffer or groundwater (Figure 1.3.2) ($P = 0.05$). Populations of *B. bifidum* in both microcosms decreased by approximately 95% after 25 h incubation (Table 1.9, appendix) and there was no significant difference in their mean decimal reduction times (Table 1.3) ($P = 0.05$).

An inversely proportional, temperature-dependent survival characteristic was shown to occur aerobically in phosphate buffer with *B. bifidum* when the survival curves at the three temperatures used in the experiment were compared (Figure 1.3.4) ($P < 0.00001$). There was also a significant difference in the decimal reduction times between all three treatments with longer decimal reduction times corresponding to lower temperatures of incubation ($P=0.05$).

1.3.3 Injury induction times. The ability of the published selective media to effectively enumerate the bifidobacterial population from a pure culture microcosm

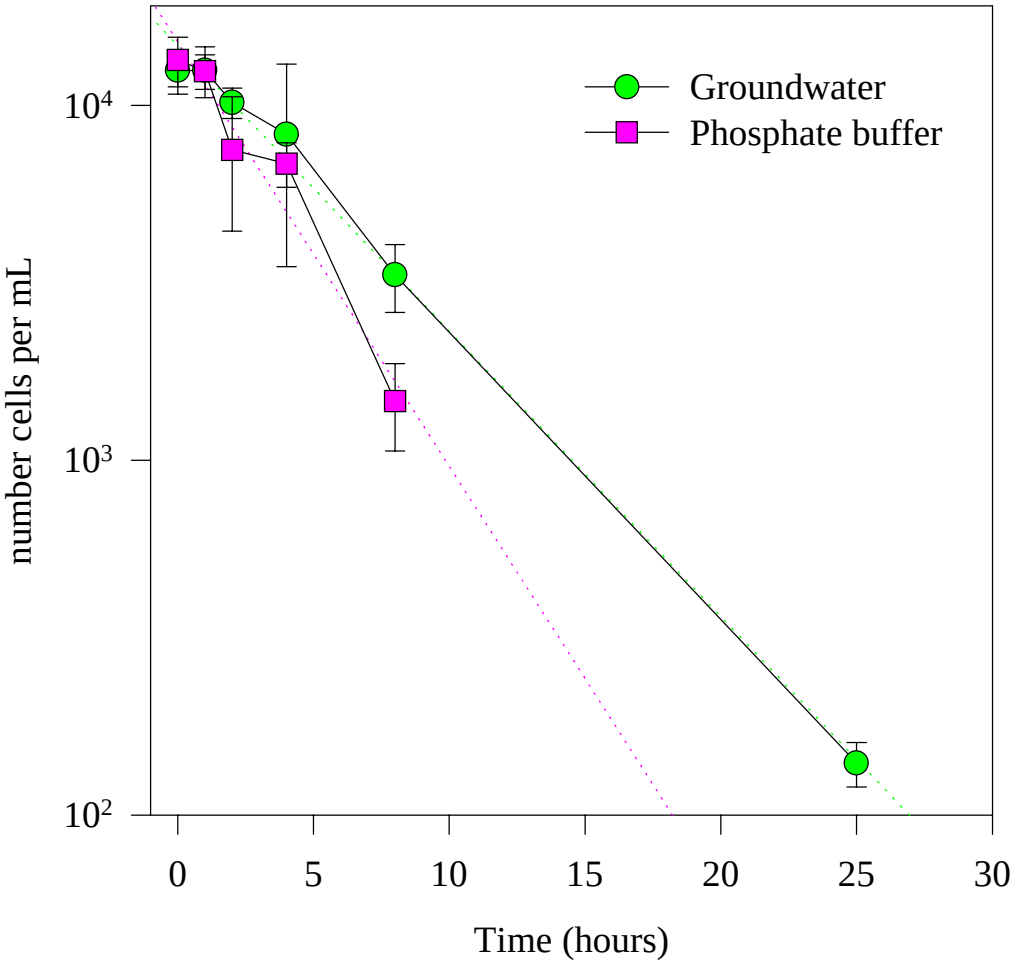

Figure 1.3.1. Aerobic survival of *B. bifidum* incubated at 35°C in sterilized groundwater and phosphate buffer microcosms and enumerated on RCA. Each data point represents the mean of three replicates. Error bars indicate the standard deviation.

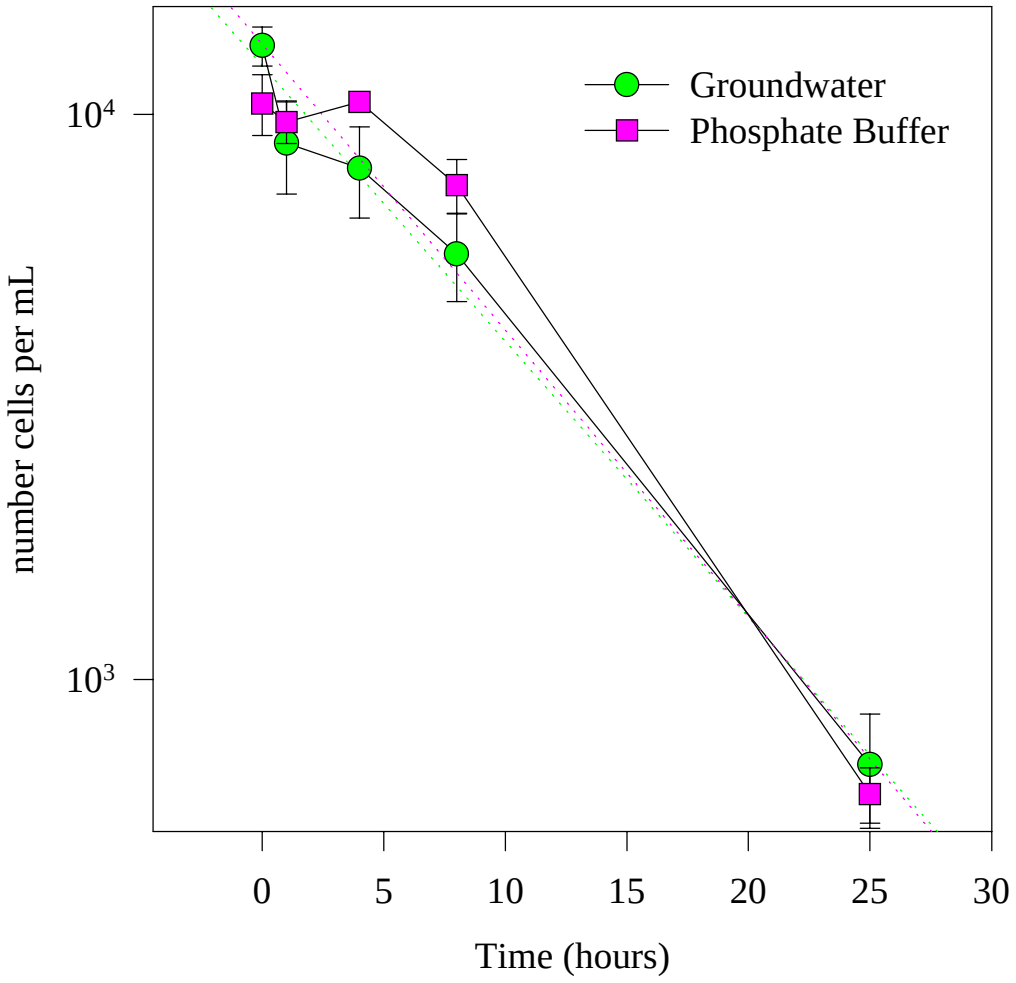

Figure 1.3.2. Aerobic survival of *B. bifidum* incubated at 28°C in sterilized groundwater and phosphate buffer microcosms and enumerated on RCA. Each data point represents the mean of three replicates. Error bars indicate the standard deviation.

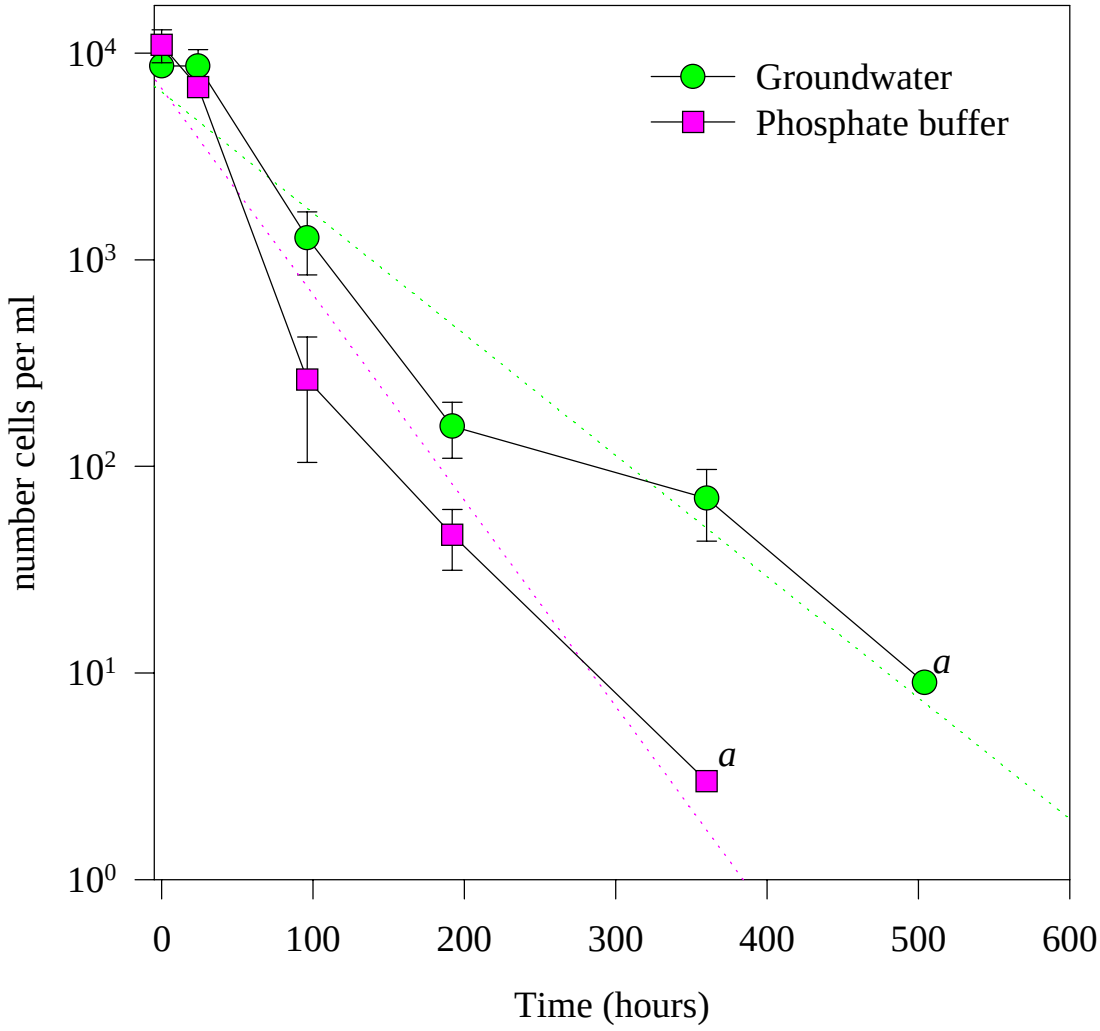

Figure 1.3.3. Aerobic survival of *B. bifidum* incubated at 13°C in sterilized groundwater and phosphate buffer microcosms and enumerated on RCA. Each data point represents the mean of three replicates. *a* = data point from 10^0 dilution; only one replicate. Error bars indicate the standard deviation.

Table 1.3. Summary of decimal reduction times (in hours) of *Bifidobacteria bifidum* in aerobic single culture microcosms incubated at 13, 28, and 35°C.[a]

Temperature °C	Decimal reduction time	
	Groundwater	Phosphate buffer
13	170.46a	100.41b
28	20.48a	19.75a
35	12.43a	8.36b

[a] Differing letters following number indicates significance between incubation media at the same temperature

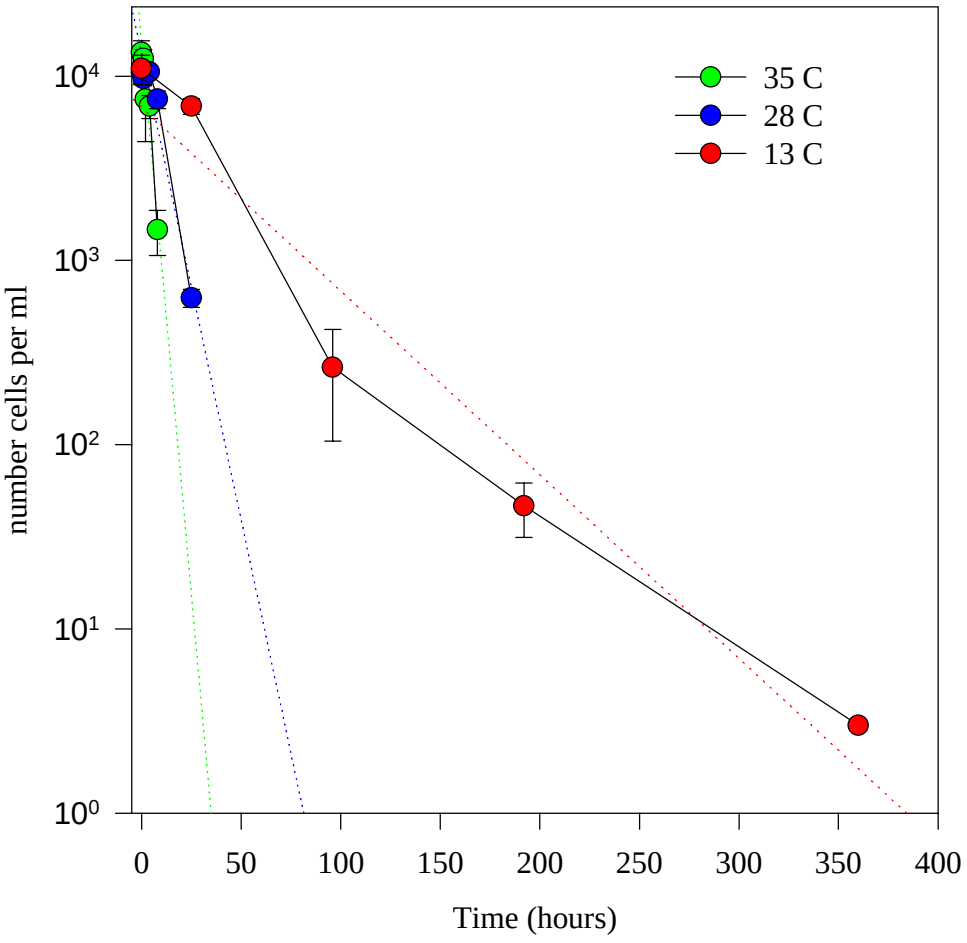

Figure 1.3.4. Comparison of aerobic survival of *B. bifidum* incubated at 13, 28, and 35°C in phosphate buffer microcosms and enumerated on RCA. Each data point represents the mean of three replicates. Error bars indicate the standard deviation.

was investigated in order to establish the extent of injury that could be expected before mixed culture microcosms were used. Injury characteristics of *B. bifidum* sampled from the aerobic microcosms and enumerated on specific selective media were investigated once the survival parameters of the species were better understood. Injured bifidobacteria were shown to occur in these experiments when enumerating the cells on B25 and YN6. RCA was used as the non-selective medium. The proportion of cells enumerated only on RCA but not the selective media from the same sample, were considered injured.

The injury induction times (IIT) for *B. bifidum* enumerated on B25 and YN6 are shown in Table 1.4. No significant differences were observed between the IITs on B25 and YN6 at 35°C. The times to reach 90 and 99% injury were significantly longer for B25 at 13°C, but there was no significant difference in the times to reach 99.9 and 99.99% injury between the two media. The IIT of YN6 were observed to be approximately twice those of B25 at 28°C and numerically greater (although not statistically greater) than the IIT of YN6 at 35°C.

The effect of the selective media B25 and B50 on the recovery of *B. bifidum* from an aerobic phosphate buffer microcosm incubated at 13°C is shown in Figure 1.3.5. RCA enumerated a significantly higher population than either B25 or B50 at the three sampling times. There was no significant difference between recoveries on B25 or B50 at zero time, but B25 did recover a significantly higher percentage of the population at later sampling times. The percentage of sublethally injured but still viable cells is shown in Table 1.5. The proportion of the population unable to grow on the selective media was high even at zero time. Over 45% of the bifidobacteria

Table 1.4. Injury induction times (IIT) in hours for *B. bifidum* incubated at 35, 28, and 13°C and enumerated on B25 and YN6 selective media.[a]

% Injury[b]	IIT					
	B25			YN6		
	35°C	28°C	13°C	35°C	28°C	13°C
90	11.4a	55.6a	123.1a	16.6a	105.9b	99.5b
99	33.3a	118.5a	245.5a	35.9a	239.5b	230.7b
99.9	55.1a	181.4a	367.8a	55.2a	373.1b	362.0a
99.99	77.0a	244.4a	490.1a	74.5a	506.7b	493.2a

[a] Differing letters following number indicates significance between selective media at the same temperature

[b] RCA was used as the nonselective reference medium

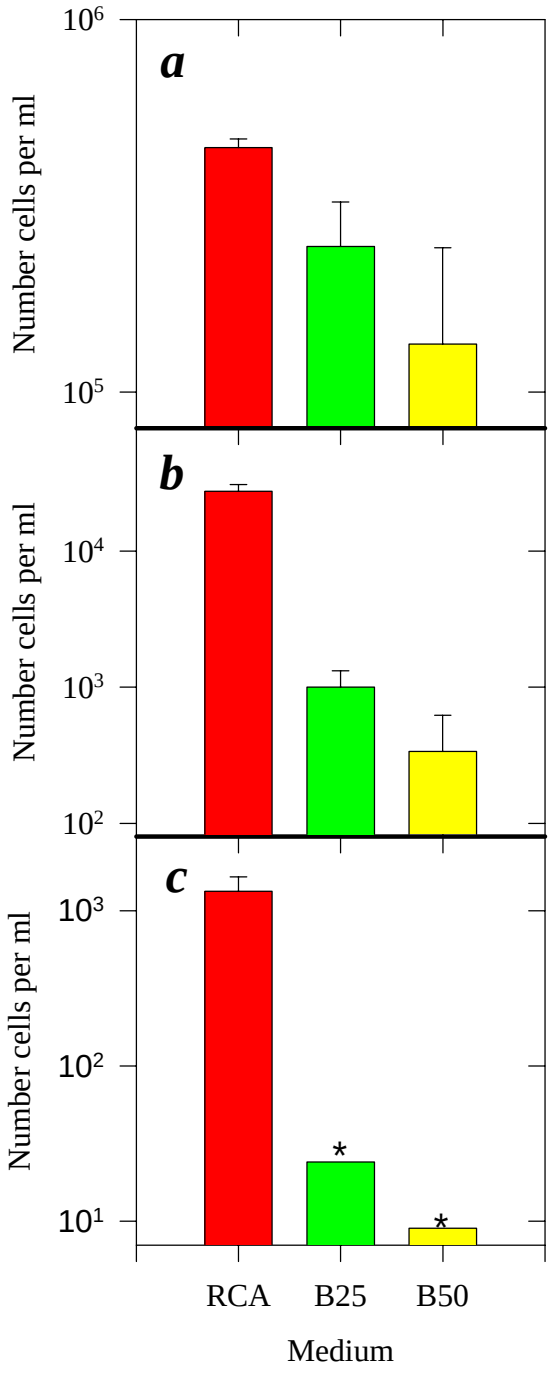

Figure 1.3.5. Aerobic survival of *B. bifidum* incubated at 13°C in sterilized phosphate buffer microcosms and enumerated on RCA, B25, and B50. Graph *a*) represents population at zero time, *b*) at 168 h, and *c*) at 264 h. Each data point represents the mean of three replicates except where noted by * indicating data for a 10^0 dilution. Error bars indicate the standard deviation.

Table 1.5. Percent injury and decrease of *B. bifidum* population incubated at 13°C in a sterilized phosphate buffer microcosm and enumerated on RCA, B25, and B50.

	Total	% Injury[b]		% Decrease[c]		
Time	Population[a]	B25	B50	RCA	B25	B50
0	4.53×10^5	45.73	70.37			
168	2.75×10^4	96.36	98.77	93.93	99.59	99.75
264	1.33×10^3	98.19	99.32	99.70	99.99	99.99

[a] as enumerated on RCA.

[b] % injury was determined for each medium by comparing selected population to the total population surviving at each sampling time.

[c] % decrease was determined by comparing the population at time *x* with the population at time zero for each medium.

enumerated on RCA did not appear on B25. That number grew to over 70 % on B50 -- a medium differing from B25 only by a doubling of the concentration of iodoacetic acid. B25 and B50 exhibited approximately 96 and 99 % injury, respectively, after incubation for 168 h. This is especially troublesome from a monitoring standpoint since the viable population of bifidobacteria as enumerated on RCA decreased 93.9 % from the starting population.

1.3.4 Aerobic mixed culture microcosms. The previous experiments documented the survival behavior of a pure inoculum of bifidobacteria subjected to temperature, oxygen, and osmotic stresses, and how those stresses affected enumeration efficiencies of bifidobacteria from the microcosms on RCA. To better simulate a more natural situation, another organism, *E. coli* ATCC 23559 was also inoculated into the microcosms and enumerated concurrently with the bifidobacteria. The purpose here was to address concerns in the literature of bifidobacterial survival compared to other indicator organisms in aquatic environments (16,42). Of concern in these experiments was the question of how efficiently the selective media would perform in enumeration of bifidobacteria both in the presence of another organism and in the absence of a non-selective enumeration medium. Essentially the same conditions were followed as in the aerobic pure culture microcosm experiments with the exception of the exclusion of RCA because of its non-selective nature. Keep in mind, however, that the use of the selective media in these experiments has been shown to underestimate the actual bifidobacteria population and that the survival of the bifidobacteria in these situations is probably somewhat longer as compared to the pure culture microcosms.

The aerobic survival of *B. bifidum* and *E. coli* incubated at 35°C in a phosphate buffer microcosm is shown in Figure 1.3.6. It is interesting to note the initial increase in bifidobacterial populations between time zero and the 4 hour sampling time. [Note: This phenomenon was also observed in the pure microcosm experiments in the case of *B. longum* incubated in groundwater at 13°C (Figure 1.5.4, appendix)]. The 0 and 4 h points were significantly different from each other for the B25 medium but not the YN6 medium (P = 0.05). This effect is probably due to the initial osmotic shock of the cells going from the growth medium to the microcosm and the slight increase being indicative of an acclimation by the remaining viable cells at this relatively high incubation temperature. This may explain why this increase was not observed in the 13 and 25°C mixed culture microcosms as shown later in this chapter. This artifact may have also skewed the actual DRT for both YN6 and B25. If one uses the 4, 5.5, 10 and 18 hour (which gave a zero population for bifidobacteria) data points to calculate the DRT, then it falls very close to the pure culture microcosm experiments. An unexpected result was the survival ability of *E. coli* in this microcosm. It was expected that of the three temperatures, the population of *E. coli* would decrease at 35°C. However, after 25 hours at 35°C, there was no decrease in the population. This would seem to contradict the work of Evison and James (17) and confirm the findings of Resnick and Levin (42).

The aerobic survival of *B. bifidum* and *E. coli* in a phosphate buffer microcosm incubated at 28°C is shown in Figure 1.3.7. Survival of *E. coli* was again longer than expected with a significantly higher calculated DRT of 369.69 h (P = 0.05). There were no observed or statistical differences in the survival or the DRT of

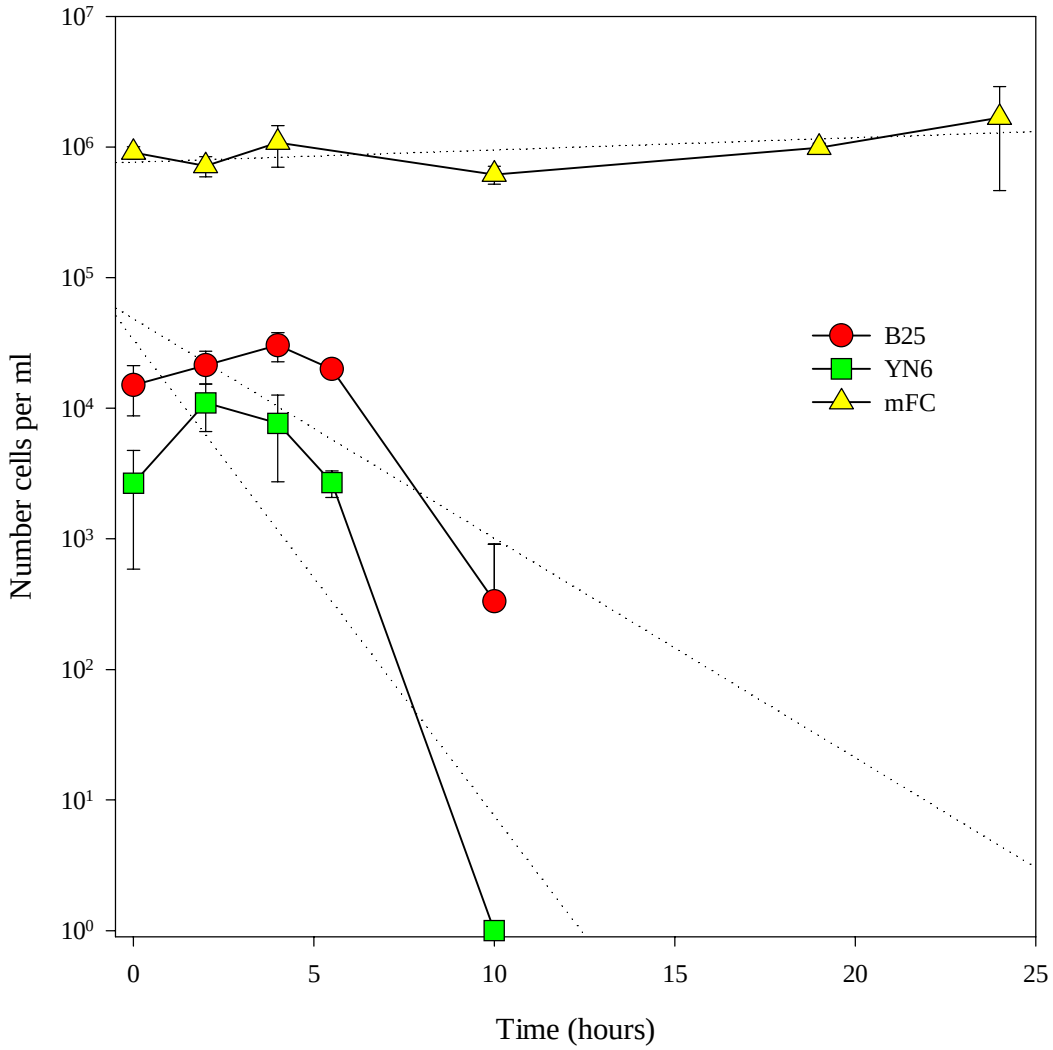

Figure 1.3.6. Aerobic survival of *B. bifidum* and *E. coli* incubated at 35°C in a phosphate buffer microcosm. Bifidobacteria were enumerated on B25 and YN6 agar and *E. coli* was enumerated on mFC agar. Each data point represents the mean of three replicates. Error bars indicate the standard deviation.

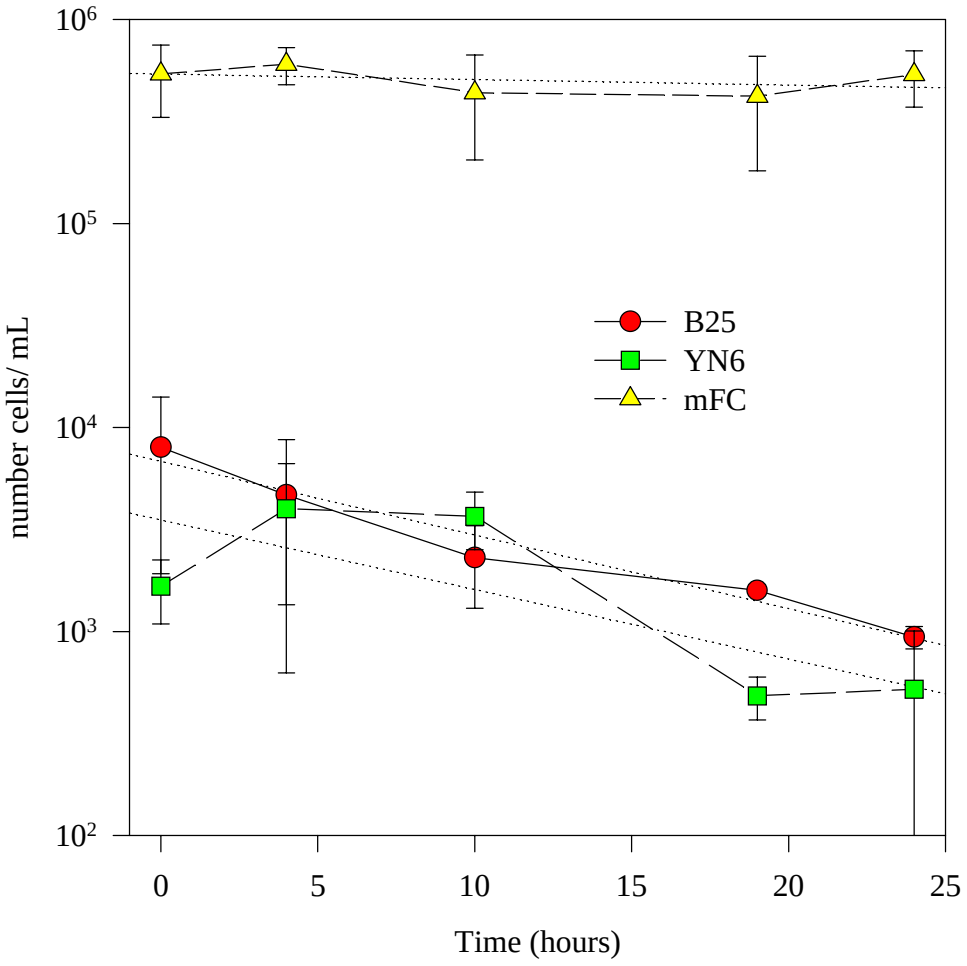

Figure 1.3.7. Aerobic survival of *B. bifidum* and *E. coli* incubated at 28°C in a phosphate buffer microcosm. Bifidobacteria were enumerated on B25 and YN6 agar and *E. coli* was enumerated on mFC. Each data point represents the mean of three replicates. Error bars indicate the standard deviation.

B. bifidum on either B25 or YN6.

In the 13°C mixed microcosm (Figure 1.3.8), similar differences in survival were observed. Both the survival and the DRT for *E. coli* were significantly longer than *B. bifidum*. However, the recovery of *B. bifidum* on B25 was significantly higher than on YN6 and the DRT on B25 was a significantly longer 111.50 h compared to 61.13 h on YN6 ($P = 0.05$). The decimal reduction times for all three incubation temperatures are summarized in Table 1.6.

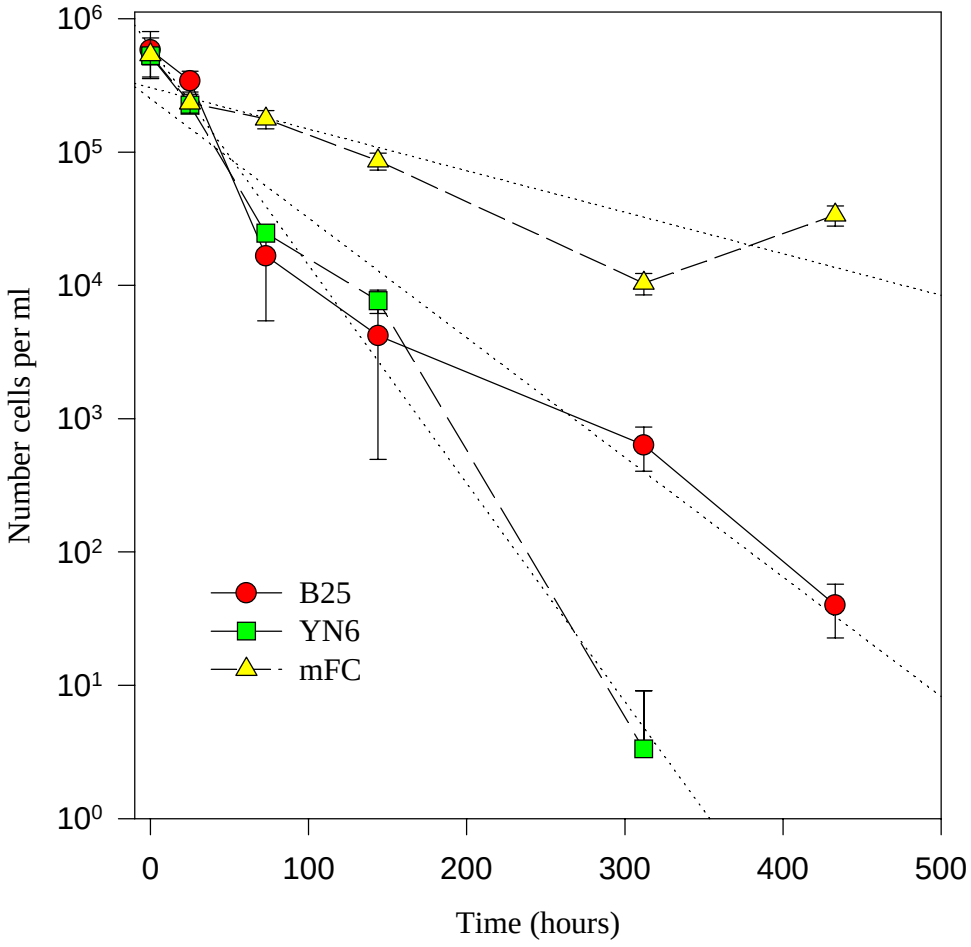

Figure 1.3.8. Aerobic survival of *B. bifidum* and *E. coli* incubated at 13°C in a phosphate buffer microcosm. Bifidobacteria were enumerated on B25 and YN6 agar and *E. coli* was enumerated on mFC agar. Each data point represents the mean of three replicates. Error bars indicate the standard deviation.

Table 1.6. Summary of decimal reduction times (in hours) of *B. bifidum* and *E. coli* in aerobic mixed culture microcosms incubated at 13, 28, and 35°C.[a]

Temperature (°C)	Decimal reduction time		
	B. bifidum		*E. coli*
	B25	YN6	mFC
13	111.50a	61.13b	320.98c
28	27.69a	29.32a	369.69b
35	5.62a	2.73b	(120.78)c[b]

[a] Differing letters following number indicates significance among selective media at the same temperature

[b] Numbers in parentheses denote negative numbers which indicates growth was occurring in the microcosm.

DISCUSSION

Using bacterial indicator organisms to monitor the quality of drinking and recreational waters has proven useful in protecting the public health. Various indicator organisms, such as total coliforms, fecal coliforms, fecal streptococci, and HPC bacteria have been used. Total coliforms have been widely used for nearly a century to ensure potability of water supplies. Fecal coliforms, a thermo-tolerant subset of total coliforms, have been used to show recent fecal contamination. Fecal streptococci, which include the enterococcus subgroup, have been used to better evaluate the source of contamination. The enterococci are mainly excreted by humans and exhibit comparable survival characteristics to total coliforms and fecal coliforms in water (56). HPC bacteria have indicated fecal contamination due to the increased number of heterotrophs observed in a contaminated sample.

Although these groups have proven valuable in ensuring safe water supplies, they all are less than ideal for indicating fecal contamination in all water sources. Total coliform bacteria can exhibit aftergrowth in waters containing organic matter and under moderate temperatures. High background bacterial populations can mask total coliform counts resulting in a dangerous overestimation of water quality. Many total coliform bacteria have been shown to be from an environmental source rather than of fecal origin. Similar problems have been shown to occur with the fecal coliforms in tropical waters (14). High numbers of fecal coliforms alone do not necessarily indicate fecal pollution and, hence, should not be used as a sole indicator

organism. Likewise, high HPC counts may indicate a high level of organic matter in water and not fecal pollution.

Bifidobacteria have been proposed as indicators of fecal pollution in water, but lack the necessary reliability and developed methodology found in the other indicator organisms. Bifidobacteria are exclusively of fecal origin, excreted in very high numbers, do not exhibit aftergrowth outside the host, and some species are exclusive to humans. These advantages make bifidobacteria an attractive choice to use as a supplemental test to monitor water quality. However, there is a lack of data describing the behavior of bifidobacteria in aquatic environments (28). What data are published often show conflicting results. Accordingly, the purpose the present study was to better characterize the survival and injury parameters of bifidobacteria in controlled aerobic aquatic microcosms. In addition, published selective media were evaluated for their recovery efficiencies to test the reliability and methodology issues. (Note: Limited experiments were completed using *B. longum* in the microcosms. Also, limited experiments with anaerobic microcosms containing *B. bifidum* were conducted. Conclusions about these experiments are included in this discussion where appropriate for comparison purposes. The reader is referred to the appendix for data presentations of these experiments.)

The first question addressed in this study was the survival characteristics of bifidobacteria in an external aquatic environment. A human-specific species, *B. bifidum*, was used in these experiments since one of the potential advantages of bifidobacteria as indicator organisms is to aid in distinguishing the source of fecal pollution. In addition, the underlying aim of these experiments was to pose a worst

case scenario for the survival of the bifidobacteria. To that end, initial assays investigated their survival in sterilized groundwater versus phosphate buffer microcosms. The type of microcosm that produced the worst survival rates would then be used in the rest of the procedures in this study.

B. longum initially showed no differences in its survival properties in phosphate buffer or groundwater (Figure 1.5.4, appendix) so the experiments were repeated with *B. bifidum*. At both 13 and 35°C, *B. bifidum* exhibited a statistically greater survival in groundwater than in phosphate buffer. Although there was no statistical differences in the microcosm at 28°C, the survival of *B. bifidum* in groundwater was slightly higher. Therefore, in the remaining assays discussed in this chapter, *B. bifidum* was incubated in phosphate buffer microcosms. It is also interesting to point out that *B. bifidum* exhibited less robust growth characteristics compared to *B. longum* (Figures 1.5.1 and 1.5.2, appendix) and that *B. bifidum* had a final population density that was over a log lower than observed for *B. longum*.

Data gathered in these experiments tend to corroborate those of Resnick and Levin -- namely, that bifidobacterial survival is poor in an aquatic environment (43). In none of these experiments was there any indication of aftergrowth. Indeed, it was difficult at the beginning to predict accurate sampling times in the microcosms incubated at 28 and 35°C. At 35°C, no viable bifidobacteria could be recovered as enumerated on the nonselective RCA in as little as 24 h from the phosphate buffer microcosms (Figure 1.3.1). Even when the microcosm was kept anaerobic throughout the incubation -- an environment more conducive to the anaerobic nature of this genus -- *B. bifidum* was unrecoverable after 27 h (Figure 1.5.6, appendix).

The second question addressed in this study was the survivability of bifidobacteria in aquatic environments compared to *E. coli*. Conflicting studies have been published. Some studies have shown bifidobacteria to outlast *E. coli* or *E coli* survival surpassing bifidobacteria (17, 28). In this current study, only stationary phase cells were used to inoculate the microcosms since exponential phase cells tend to be more sensitive to external stresses. Bifidobacteria as enumerated on nonselective RCA exhibited a significant reduction in the viable population in as little as approximately 24 hours post-inoculation for the 35°C aerobic microcosms. There was no convincing evidence of aftergrowth of bifidobacteria as enumerated on RCA. Experiments that suggested aftergrowth were in the 35°C mixed culture microcosms when bifidobacteria were enumerated only on selective media. This may actually show a cellular repair phenomenon at work since the increase in cell numbers was short lived (less than 5 hours) and did not occur when bifidobacteria were enumerated on RCA in the single culture microcosms. In addition, *E. coli* did not exhibit a significant decrease in population over the course of the experiments regardless of the temperature of incubation or anoxic conditions (Figure 1.5.7, appendix).

The poor survival of bifidobacteria in an external aquatic environment was unambiguous in these experiments. A potential advantage for including bifidobacteria in water quality assessment would be the indication of recent human contamination of a water supply. One parameter not addressed in this study was the presence of organic matter. It would be interesting to see if high organic matter levels change the survival of bifidobacteria under similar conditions.

One major problem with using bifidobacteria as indicator organisms arose during the course of this study. The inconsistency in the results gathered from the selective media was quite disturbing. At first, it was thought that this was due to the time consuming and complex demands of preparing the media and then the subtle differences in the plating techniques of some of the media (i.e., overlaying the selective agents onto a nonselective base) combined to produce mercurial results. However, after numerous attempts and much attention paid to quality control, the inconsistencies can be attributed to a needlessly elaborate preparation regime and errors in the published recipes. Although a more detailed discussion of this problem follows, the most glaring obstacle in the preparation of the selective media can be seen in the research reported by Resnick and Levin (42,43) which details the use of YN6 in the enumeration of bifidobacteria from aquatic sources. Nowhere in the paper is there a mention of a carbon source in the medium formulation itself. Only in the discussion is there any clue as to what was used as a carbon source -- lactose -- but not in what amount. To address this omission, the author arbitrarily used lactose in a concentration (g/l) equal to that of glucose in the non-selective RCA formulation. While such an oversight on the part of the authors may seem trivial, it does unnecessarily complicate any attempt to standardize a methodology for using this genus as an indicator organism. Standardization is most important from a regulatory standpoint as health, policy and judicial decisions are based on solid, demonstrative data collection and justifiable, *reliable* results. The exclusion of such an integral component of the selective medium -- itself the focal point of that research and a follow-up studies -- is a regrettable error.

An illustration of that inconsistency in the selective media can be shown in the following example. It is interesting to note that while B25 initially appeared to be less inhibitory than the YN6 medium to a fresh inoculum of bifidobacteria, B25 quickly became toxic to the vast majority of cells which were injured but still viable in the microcosms, even with the 5 h nonselective RCA recovery step included in the enumeration technique. *B. bifidum* cells utilized in the microcosms were inoculated from a culture at the beginning of stationary phase at an approximate age of 100 to 120 h, so physiological reasons, such as marked peptidoglycan deterioration or secondary metabolite toxicity, do not seem to account for the severe inability of the cells to grow on either selective medium, even at zero time. Oxygen toxicity could account for only some of the injury at zero time since the cells were spread-plated and incubated anaerobically without delay after the initial microcosm inoculation and the recovery step for B25 should have allowed enough time -- almost a complete generation cycle -- for most injured cells to repair cellular structures, such as membranes and inactivated enzymes, before they were exposed to the inhibitory components of the selective agar overlay. It would appear, therefore, that either the recovery step should be extended, in which case the selective efficiency of B25 could be compromised, or the selective agents themselves are too toxic to allow effective enumeration of a large proportion of the viable population of cells. Perhaps the addition of a free radical scavenger to the basal medium would increase the efficiency of either medium by aiding in the repair mechanisms of the injured cells. Given that this injury phenomenon exists to such an extent using these selective media, any observations made from either the mixed microcosms or any field sampling should be

viewed as an underestimation of the true bifidobacteria population and, hence, an optimistic estimation of the actual water quality. This conclusion is not a condemnation of the use of bifidobacteria as an indicator organism, but only a warning about relying on the published selective media to gauge water quality, a 40 year old hurdle which has yet to be jumped.

Another problem encountered with the present selective enumeration media was the question of reliability and reproducibility of the media from batch to batch, which became a great concern with the YN6 medium. Many plates of each medium were utilized in these experiments and each set of plates poured was rarely stored at 4°C for more than 5 days before they were used, a practice which necessitated a high frequency of media preparation. The author personally completed all selective media preparation in an attempt to minimize variation in the final medium product. Furthermore, components of the selective media were regularly replaced to ensure their freshness; this was especially true of the antibiotics and iodoacetate. Unfortunately, multiple experiments needed to be repeated a number of times to allow enough data points to be collected in order for a trend to be observed. Figures 1.3.9a and b depict one of the experiments that investigated the anaerobic survival of *B. bifidum* incubated at 13°C and enumerated on RCA, YN6, and B25. Two times are shown, zero and 96h on graph a and b, respectively. No cells were observed on the YN6 plates after 96 h even though the medium proved to be better able to enumerate bifidobacteria over time compared to B25 at 35 and 28°C (Figures 1.3.9c and d and 1.3.10). Due to these reliability issues with the performance of YN6 in these experiments, as well as the mixed microcosm experiments also discussed in this

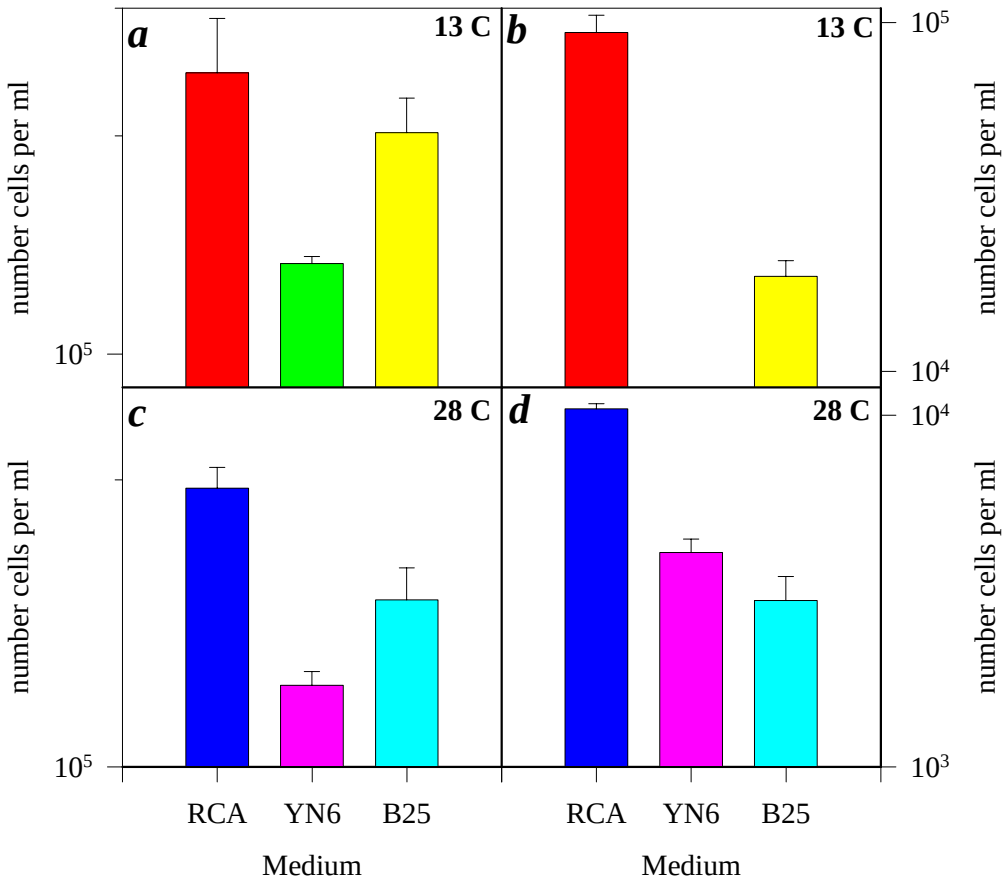

Figure 1.4.1. Anaerobic survival of *B. bifidum* incubated in sterilized phosphate buffer microcosms and enumerated on RCA, YN6, and B25. Graph a) zero time and 13°C, b) 96 h and 13°C, c) zero time and 28°C, and d) 27 h and 28°C. Each data point represents the mean of three replicates. Error bars indicate the standard deviation.

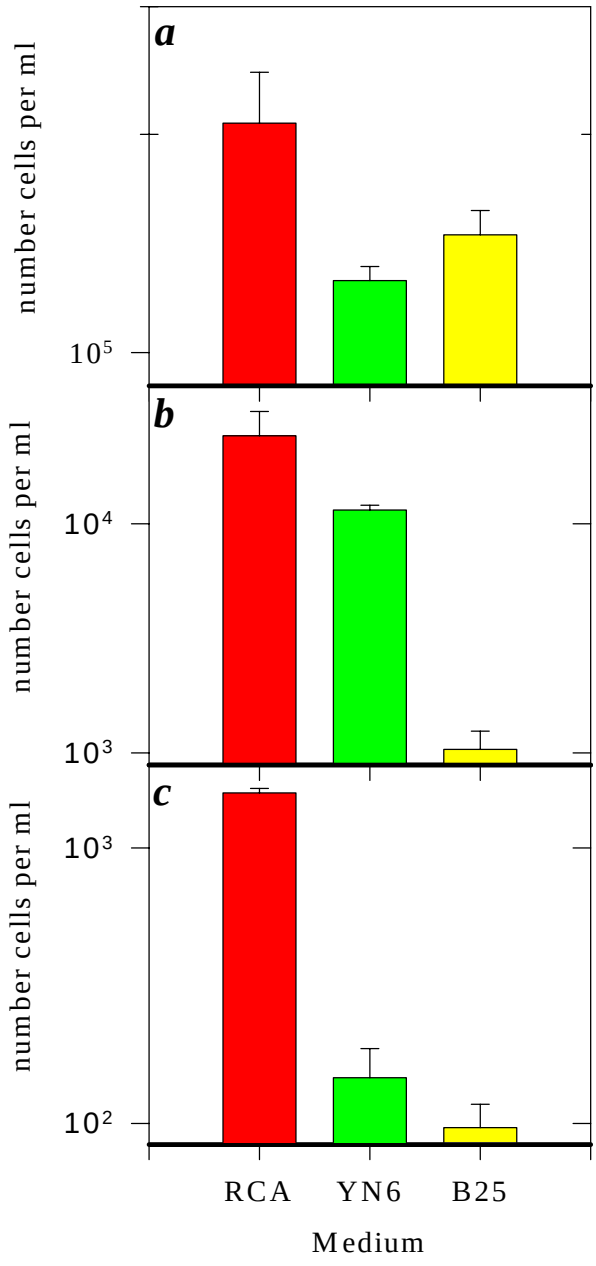

Figure 1.4.2. Anaerobic survival of *B. bifidum* incubated at 35°C in sterilized phosphate buffer microcosms and enumerated on RCA, YN6, and B25. Graph a) represents population at zero time, b) at 8 h, and c) at 14 h. Each data point represents the mean of three replicates. Error bars indicate the standard deviation.

chapter, YN6 is not recommended to effectively enumerate bifidobacteria from aquatic systems.

Bifidobacteria, as fecal pollution indicators, appear to satisfy some of the ideal indicator organism criteria. Results of the present study have shown bifidobacteria maintain a population sufficient to allow detection in microcosms for a short time and they are incapable of aftergrowth in external aquatic environments low in organic matter even when those aquatic environments are anoxic. The survival of the bifidobacteria population was indirectly proportional to the temperature at which the microcosm was stored. The lack of an acceptable enumeration medium continues to be a major disadvantage in trying to enumerate bifidobacteria from water samples. Selective media found in the literature were shown to exhibit insufficient sensitivity to effectively enumerate bifidobacteria. In mixed microcosm studies with *E. coli*, the survival of the bifidobacteria population was considerably lower than that of the *E. coli* population. Therefore, detection of bifidobacteria in a water sample would indicate a fairly recent – and possibly human – contamination event. Detection of higher densities of bifidobacteria in water samples could demonstrate either a very recent contamination or ongoing contamination, such as percolation of leachate from a septic system or the effluent of a sewage outflow. *E. coli* did not exhibit a significant reduction in population in the microcosms at any of the temperatures used in the experiments. This would corroborate the work done by Carrillo et al. (14) who suggested that coliforms have the ability to adapt to and become part of the natural aquatic microflora. While these organisms may have had a fecal source originally, that may not mean that their presence indicates recent or human contamination. The

regulatory implications of these facts may have significant health and economic impacts especially in rural and tropical areas.

Appendix

Methods and Materials

1.5.1. Aerobic pure culture survival experiments with *B. longum*. All procedures used in these experiments were the same as those used with *B. bifidum*.

1.5.2. Anaerobic pure culture survival experiments. As in the aerobic pure culture experiments, frozen inocula of *B. bifidum* were used to inoculate RCM and incubated anaerobically for 96 h at 35°C. One ml of the culture was then used to separately inoculate an anaerobic 300 ml side arm flask containing 100 ml of phosphate buffer supplemented with 50 mg cysteine hydrochloride, 0.38 ml 10N NaOH, and approximately 0.05 ml rezazurin stock solution. Flasks were stored at 13, 28, or 35°C.

Anaerobic conditions were achieved by autoclaving the side arm flasks followed by cooling under CO_2. Upon reaching room temperature, the flasks were sealed by tightening the screw cap and applying black electrical tape around the neck of the flask and rubber stopper and then overpressurized (about 2 psi). When inoculating or sampling via the side arm tube, the flasks were sparged with CO_2 injected through the Balch stopper, sealed, and overpressurized again. Samples were diluted appropriately, spread-plated in triplicate on RCA, and incubated anaerobically at 35°C for 72 h. Resulting colonies were counted and the replicates averaged.

1.5.3. Anaerobic mixed culture survival experiments. As in the aerobic mixed culture experiments, *B. bifidum* and *E. coli* were used to inoculate 300 ml side arm flasks containing 100 ml phosphate buffer supplemented with 50 mg cysteine hydrochloride, 0.38 ml 10N NaOH, and approximately 0.05 ml rezazurin stock

solution. Anaerobic conditions in the flasks were achieved and maintained according to the same procedure as in the anaerobic pure culture experiments. Samples were removed at various times, appropriately diluted, and spread plated in triplicate onto B25, YN6 and mFC agar. All plates except the mFC plates were incubated anaerobically at 35°C. All mFC plates were incubated aerobically at 44.5°C for 24 h.

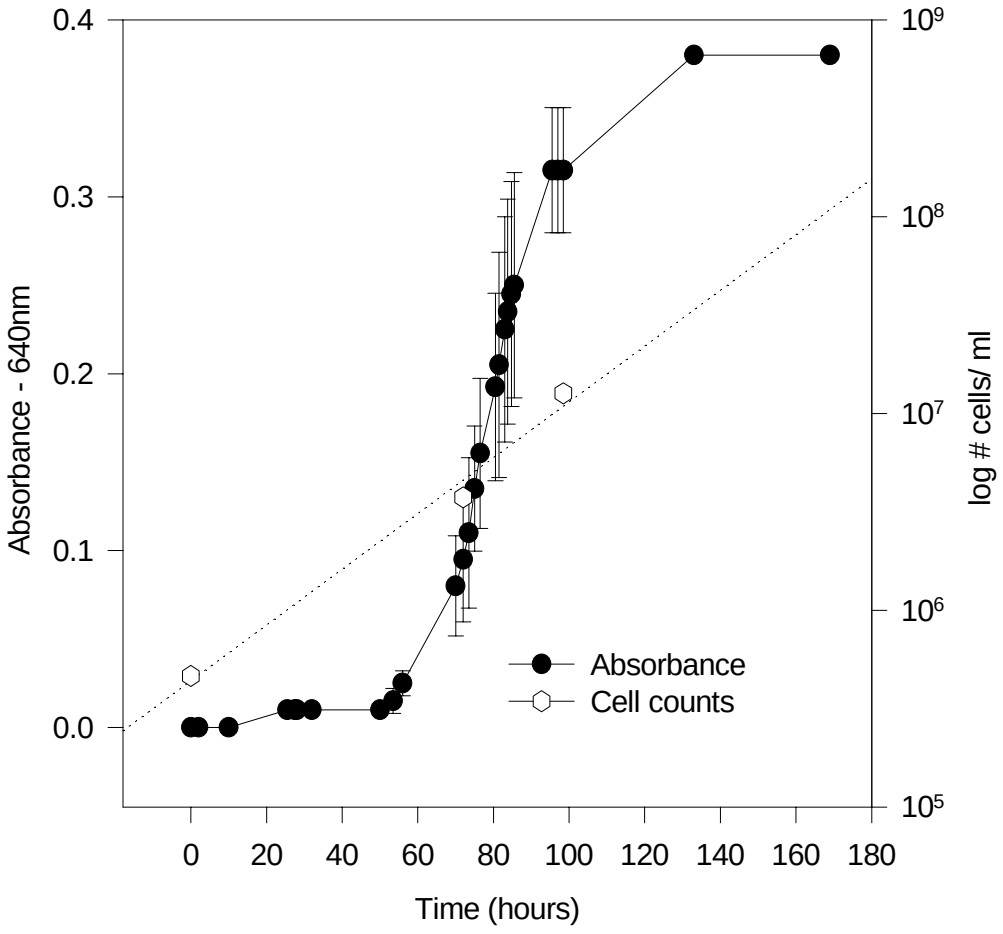

Figure 1.5.1. Growth curve for *B. bifidum*. Each data point represents the mean of three replicates. Error bars indicate the standard deviation.

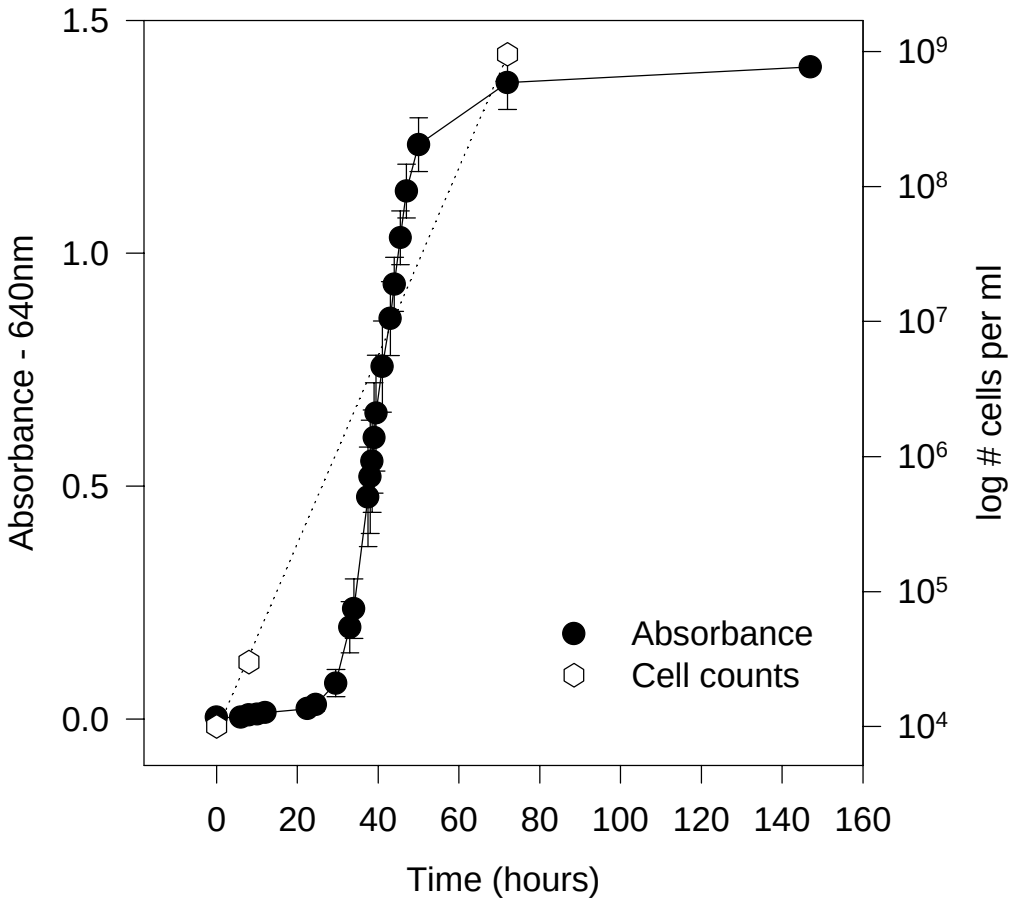

Figure 1.5.2. Growth curve for *Bifidobacterium longum*. Each data point represents the mean of three replicates. Error bars indicate the standard deviation.

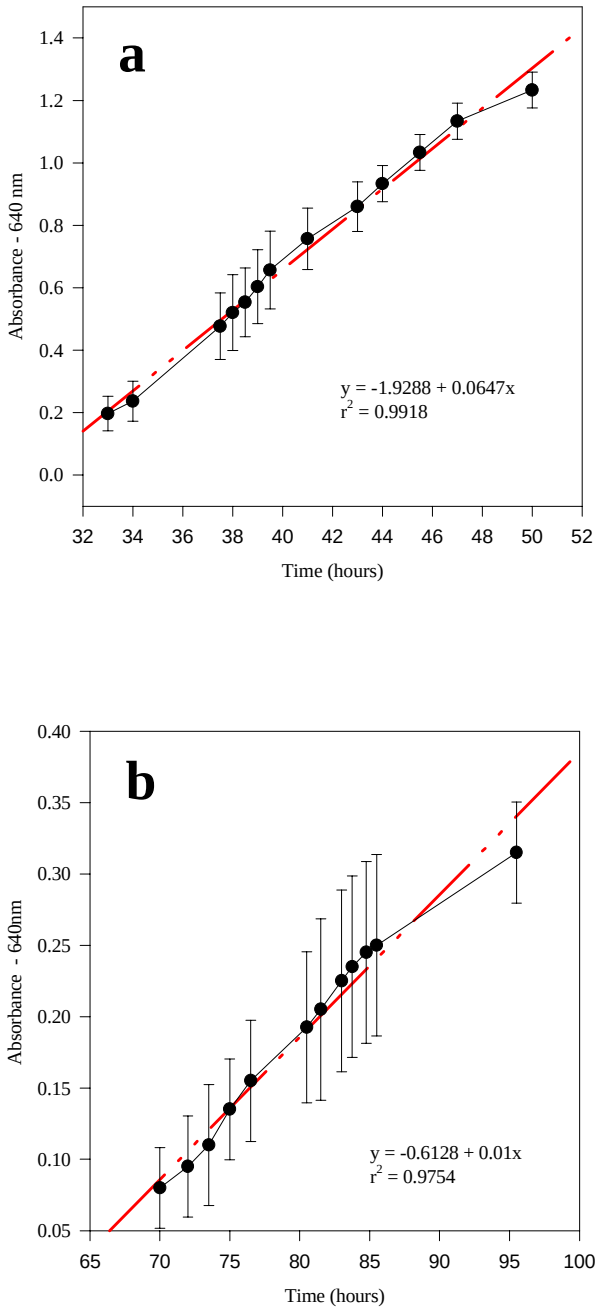

Figure 1.5.3.a and 1.5.3.b. Determination of μ value from log phase of growth for (a) *B. longum* and (b) *B. bifidum*. Error bars indicate the standard deviation.

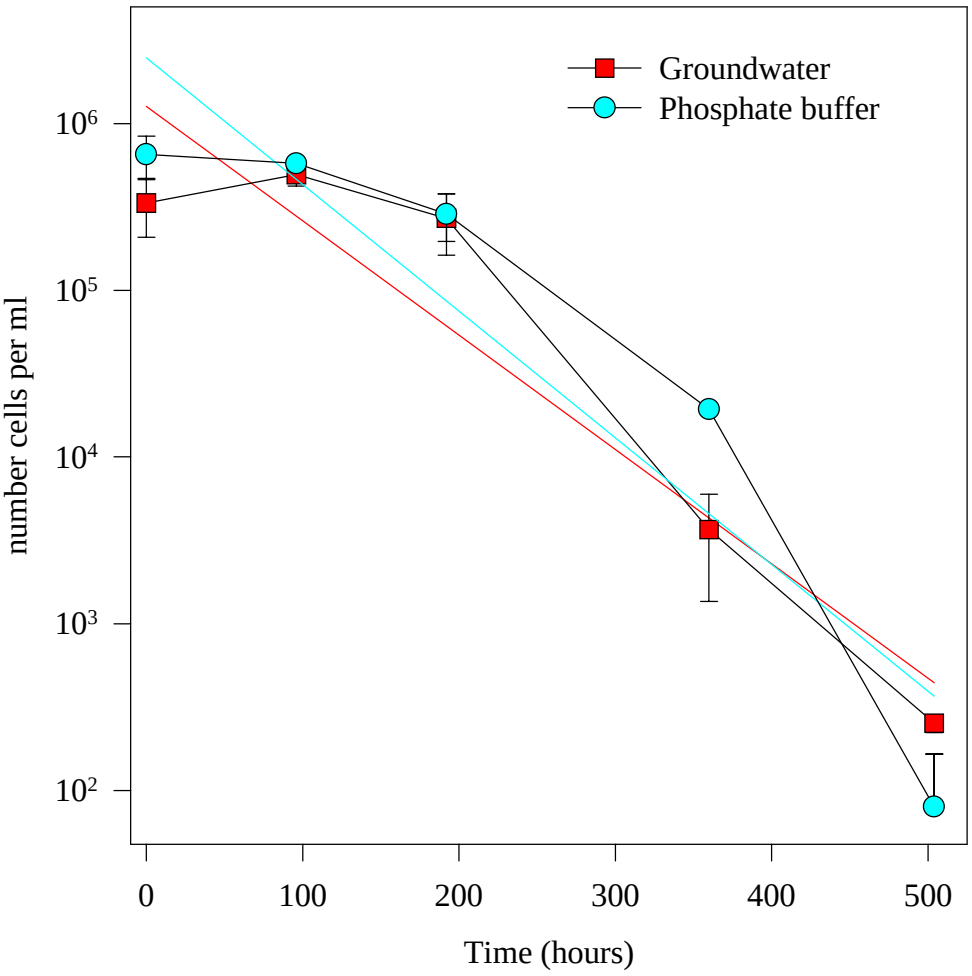

Figure 1.5.4. Aerobic survival of *B. longum* incubated at 13°C in sterilized groundwater and phosphate buffer microcosms and enumerated on RCA. Each data point represents the mean of three replicates. Error bars indicate the standard deviation.

Table 1.7. Actual and relative numbers of *B. longum* incubated at 13°C in different microcosms.

Time	Mean # cells per ml of		% Decrease		Mean decimal reduction times	
	Ground-water	Phosphate buffer	Ground-water	Phosphate buffer	Ground-water	Phosphate buffer
0	3.33×10^5	6.55×10^5	--	--		
96	4.93×10^5	5.77×10^5	(48)	11.91		
192	2.70×10^5	2.87×10^5	18.92	56.18	145.81	131.69
360	3.67×10^3	1.93×10^4	98.90	97.05		
504	2.53×10^2	8.00×10^1	99.92	99.99		

Table 1.8. Actual and relative numbers of *B. bifidum* incubated at 35°C in different microcosms.

Time	Mean # cells per ml of		% Decrease		Mean decimal reduction times	
	Ground-water	Phosphate buffer	Ground-water	Phosphate buffer	Ground-water	Phosphate buffer
0	1.26×10^4	1.34×10^4	--	--		
1	1.26×10^4	1.25×10^4	0.00	6.95		
2	1.02×10^4	7.50×10^3	18.83	44.17	12.43	8.36
4	8.30×10^3	6.87×10^3	33.95	48.88		
8	3.33×10^3	1.47×10^3	73.47	89.08		
25	1.40×10^2	ND[a]	98.89	100.00		

[a] ND not detected

Table 1.9. Actual and relative numbers of *B. bifidum* incubated at 28°C in different microcosms.

Time	Mean # cells per ml of		% Decrease		Mean decimal reduction times	
	Ground-water	Phosphate buffer	Ground-water	Phosphate buffer	Ground-water	Phosphate buffer
0	1.32×10^4	1.05×10^4				
1	8.90×10^3	9.70×10^3	32.75	7.32		
2	8.03×10^3	1.05×10^4	39.29	-0.64	20.48	19.75
4	5.67×10^3	7.50×10^3	57.18	28.34		
25	7.07×10^3	6.27×10^2	94.66	94.01		

Table 1.10. Actual and relative numbers of *B. bifidum* incubated at 13°C in different microcosms.

Time	Mean # cells per ml of		% Decrease		Mean decimal reduction times	
	Ground-water	Phosphate buffer	Ground-water	Phosphate buffer	Ground-water	Phosphate buffer
0	8.67×10^3	1.10×10^4				
24	8.67×10^3	6.87×10^3	0.00	37.58		
96	1.28×10^3	2.63×10^2	85.27	97.61	170.46	100.41
192	1.57×10^2	4.67×10^1	98.19	99.58		
360	7.00×10^1	3.00×10^0	99.19	99.97		
504	9.00×10^0	ND[a]	99.90	100.00		

[a] ND not detected

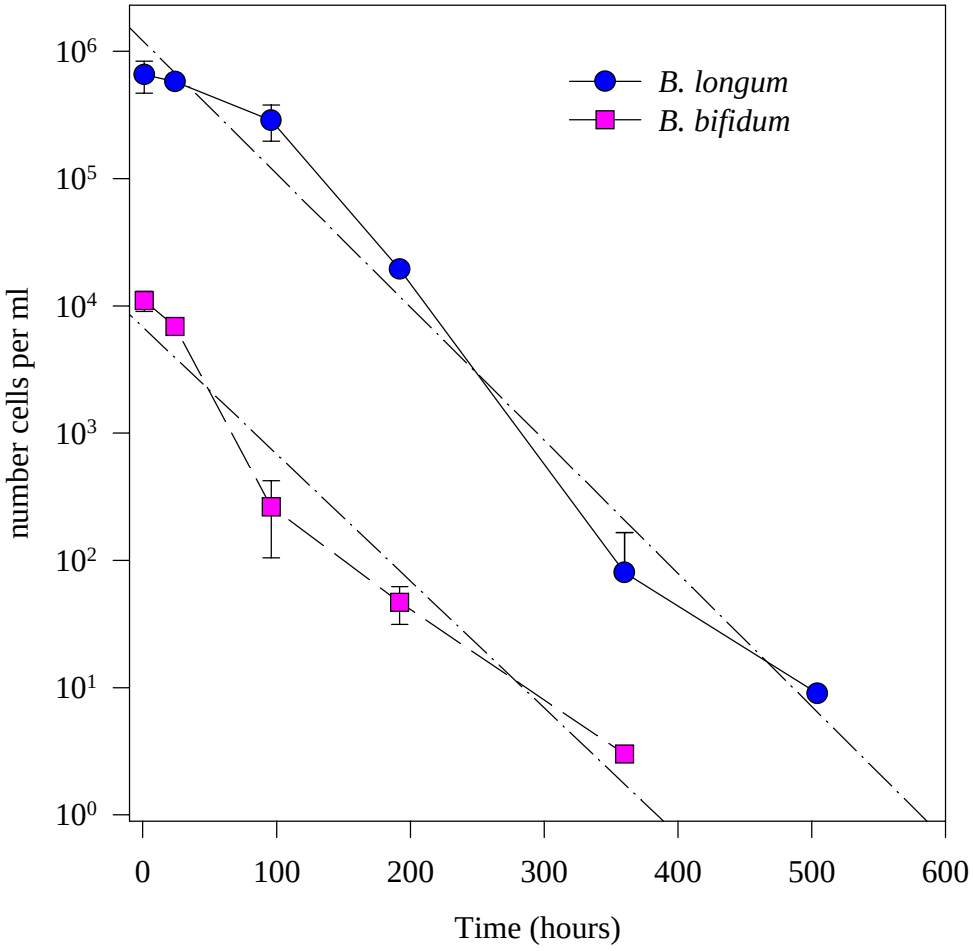

Figure 1.5.5. Comparison of aerobic survival of *B bifidum* and *B. longum* incubated at 13°C in phosphate buffer microcosms and enumerated on RCA. Each data point represents the mean of three replicates. Error bars indicate the standard deviation.

Table 1.11. Actual and relative numbers of *B. bifidum* and *E. coli* incubated at 35°C in aerobic microcosms.

Time (hr)	Mean # cells per ml of			% Decrease			Decimal Reduction		
	B25	YN6	mFC	B25	YN6	mFC	B25	YN6	mFC
0	1.50×10^4	2.67×10^3	9.07×10^5						
4	3.03×10^4	7.67×10^3	1.08×10^6	-102	-187.5	-19.49			
10	3.33×10^2	1.00×10^0	6.17×10^5	97.78	99.96	31.99	5.62	2.73	-120.78
19	0	0	9.90×10^5	100	100	8.62			
24	0	0	1.68×10^6	100	100	-172.97			

Table 1.12. Actual and relative numbers of *B. bifidum* and *E. coli* incubated at 28°C in aerobic microcosms.

Time (hr)	Mean # cells per ml of			% Decrease			Decimal Reduction		
	B25	YN6	mFC	B25	YN6	mFC	B25	YN6	mFC
0	8.00×10^3	1.67×10^3	5.40×10^5						
4	4.67×10^3	4.00×10^3	6.03×10^5	41.67	-140	-11.73			
10	2.30×10^3	3.67×10^3	4.37×10^5	71.25	-120	19.14	27.69	29.32	369.69
19	1.59×10^3	4.83×10^2	4.20×10^5	65.93	87.92	30.39			
24	9.40×10^2	5.20×10^2	5.37×10^5	59.13	85.82	-22.90			

Table 1.13. Actual and relative numbers of *B. bifidum* and *E. coli* incubated at 13°C in aerobic microcosms.

Time (hr)	Mean # cells per ml of			% Decrease			Decimal Reduction		
	B25	YN6	mFC	B25	YN6	mFC	B25	YN6	mFC
0	5.83×10^5	5.27×10^5	5.37×10^5						
25	3.42×10^5	2.25×10^5	2.33×10^5	41.31	57.34	56.52			
73	1.67×10^4	2.47×10^4	1.77×10^5	97.14	95.32	67.02	111.50	61.13	320.98
144	4.20×10^3	7.67×10^3	8.57×10^4	99.28	98.54	84.04			
312	6.33×10^2	3.33	1.04×10^4	99.89	100	98.07			
433	40	0	3.37×10^4	99.99	100	93.72			

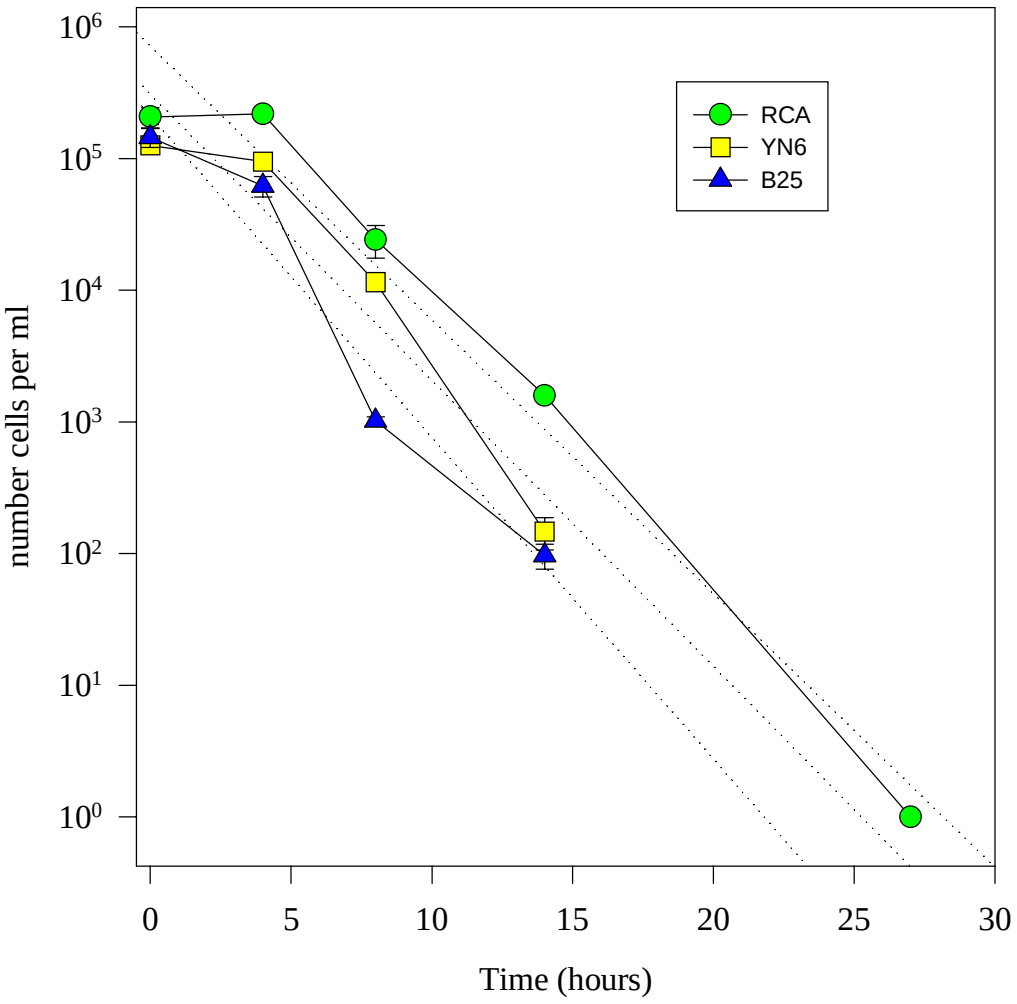

Figure 1.5.6. Anaerobic survival of *B. bifidum* 35°C in a phosphate buffer microcosm. Bifidobacteria were enumerated on RCA, B25, and YN6. Each data point represents the mean of three replicates. Error bars indicate the standard deviation.

Table 1.14. Actual and relative numbers of *B. bifidum* incubated anaerobically at 35°C in phosphate buffer microcosms plated on nonselective and selective media.

Time	Total Population[a]	% Injury[b]		% Decrease[c]		
		YN6	B25	RCA	YN6	B25
0	2.07×10^{5}	39.39	29.90			
4	2.19×10^{5}	56.55	71.65	-5.47	24.40	57.34
8	2.42×10^{4}	52.54	95.74	88.31	90.85	99.29
14	1.59×10^{3}	90.76	93.91	99.23	99.88	99.93
27	3.33×10^{-1}	100.00	100.00	>99.99	100.00	100.00

[a] as enumerated on RCA

[b] % injury was determined for each medium by comparing selected population to the total population surviving at each sampling time

[c] % decrease was determined by comparing the population at time *x* with the population at time zero for each medium

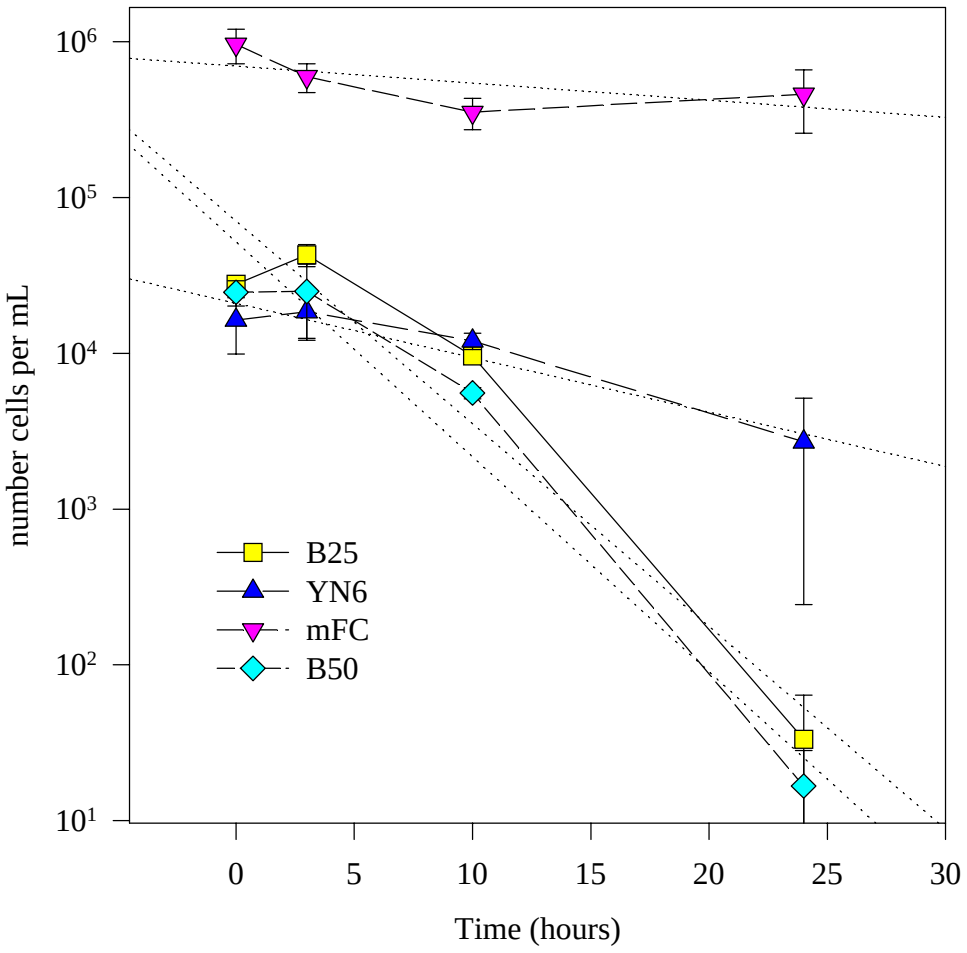

Figure 1.5.7. Anaerobic survival of *B. bifidum* and *E. coli* at 35°C in a phosphate buffer microcosm. Bifidobacteria were enumerated on B25, B50, and YN6 agar and *E. coli* was enumerated on mFC agar. Each data point represents the mean of three replicates. Error bars indicate the standard deviation.

CHAPTER 2

Occurrence of *Bifidobacterium* spp. in Constructed Wetlands Receiving Primary Treated Sewage

INTRODUCTION

Bifidobacteria have been proposed as general indicators of fecal pollution of water and, in particular, as differential indicators to distinguish human versus animal sources of pollution. They are obligate anaerobes with complex nutrient requirements and are exclusively of fecal origin. Despite these apparent advantages, there is a deficiency of data documenting the behavior of bifidobacteria in external environments such as soil, sediment, and ground- and surface-waters. This lack of attention can largely be attributed to a lack of an acceptable enumeration medium, as well as the inherent difficulty in regularly working with anaerobes.

Gyllenberg et al. (23) stressed that the importance of the specificity of bifidobacteria as indicators of fecal pollution "may depend largely upon their extra-enteral survival, particularly with the survival of other bacteria of intestinal origin, for example, coliforms and enterococci." Studies addressing the survival of bifidobacteria in surface waters have been reported (14, 17, 23, 43). However, there has been a paucity of information on the behavior of bifidobacteria in saturated soils and sediments, such as aquifers and wetlands, in which anoxic or microaerophilic conditions can occur and even prevail. This is particularly important in the United States as greater than 50 percent of the population receive their drinking water from groundwater sources and the most common water quality problem in rural water supplies stems directly from bacterial contamination from septic tanks (55). Alternative waste treatment systems such as constructed wetlands have been installed

in situations where more traditional systems have proven ineffective. Pathogens are either removed by the filtering process through the wetland matrix or are rendered non-viable by the retention time in the wetland. The microaerophilic zones found in these wetlands should allow the survival of bifidobacteria and thus permit the use of this genus as indicators of contamination of drinking water supplies. Detection of bifidobacteria in drinking water supplies possibly tainted from these systems could directly signify a human source of contamination. A more comprehensive understanding of the fate of bifidobacteria in these environments may enable a better evaluation of the quality of our nation's drinking water supplies, especially those of groundwater sources, as well as a way to pinpoint the source(s) of contamination.

The purpose of this investigation was to asses the effectiveness of published selective media (YN6, BIM25, and BIM50 agar) in monitoring the fate of bifidobacteria from constructed wetlands receiving primary treated sewage using. The specific objectives of this study were: 1) to examine the occurrence of bifidobacteria in constructed wetlands receiving primary treated sewage; 2) to evaluate published selective media for their sensitivity and selectivity for the recovery of bifidobacteria; and, 3) to observe the relative survival of bifidobacteria versus traditional indicator bacteria in constructed wetlands.

MATERIALS AND METHODS

2.2.1 Wetlands. The constructed wetlands consisted of 20 cells each receiving primary treated sewage (Figure 2.2.1). The wetlands were located at the municipal sewage treatment plant in Morgantown, West Virginia. Five different plant treatments were tested: no plants, *Scirpus* spp. (bulrushes), *Juncus* spp. (rushes), *Typha* spp. (cattails), and a mixture of the three genera. A pair of wetland cells were assigned to each plant treatment. In addition, two different depths, shallow and deep, were used for each plant treatment, thus totaling 20 wetland cells (Table 2.2.1). To postpone freezing as long as possible, the cells were wrapped with R25 fiberglass insulation. All hoses and PVC piping were also wrapped with foam insulation.

2.2.2 Sample collection. Water samples were collected from spigots installed in the bottom of the wetlands in 1 L sterile glass screw cap bottles, placed in a cooler, and transported back to the laboratory within 1 hour of collection. During collection, an effort was made to completely fill the bottles so that there was no air in the head space when the cap was replaced. Upon arrival, the samples were placed in a 13°C refrigerator until processing. Field measurements of temperature and pH were recorded for each sample upon collection.

2.2.3 Enumeration of bacteria. Enumeration of *Bifidobacterium* spp. and fecal coliforms were completed periodically for the influent and at least two plant treatments. *Bifidobacterium* spp. were initially recovered by spread plating aliquots onto YN6, B25, and B50 media (Tables 1.1, 1.2). For enumeration of bifidobacteria

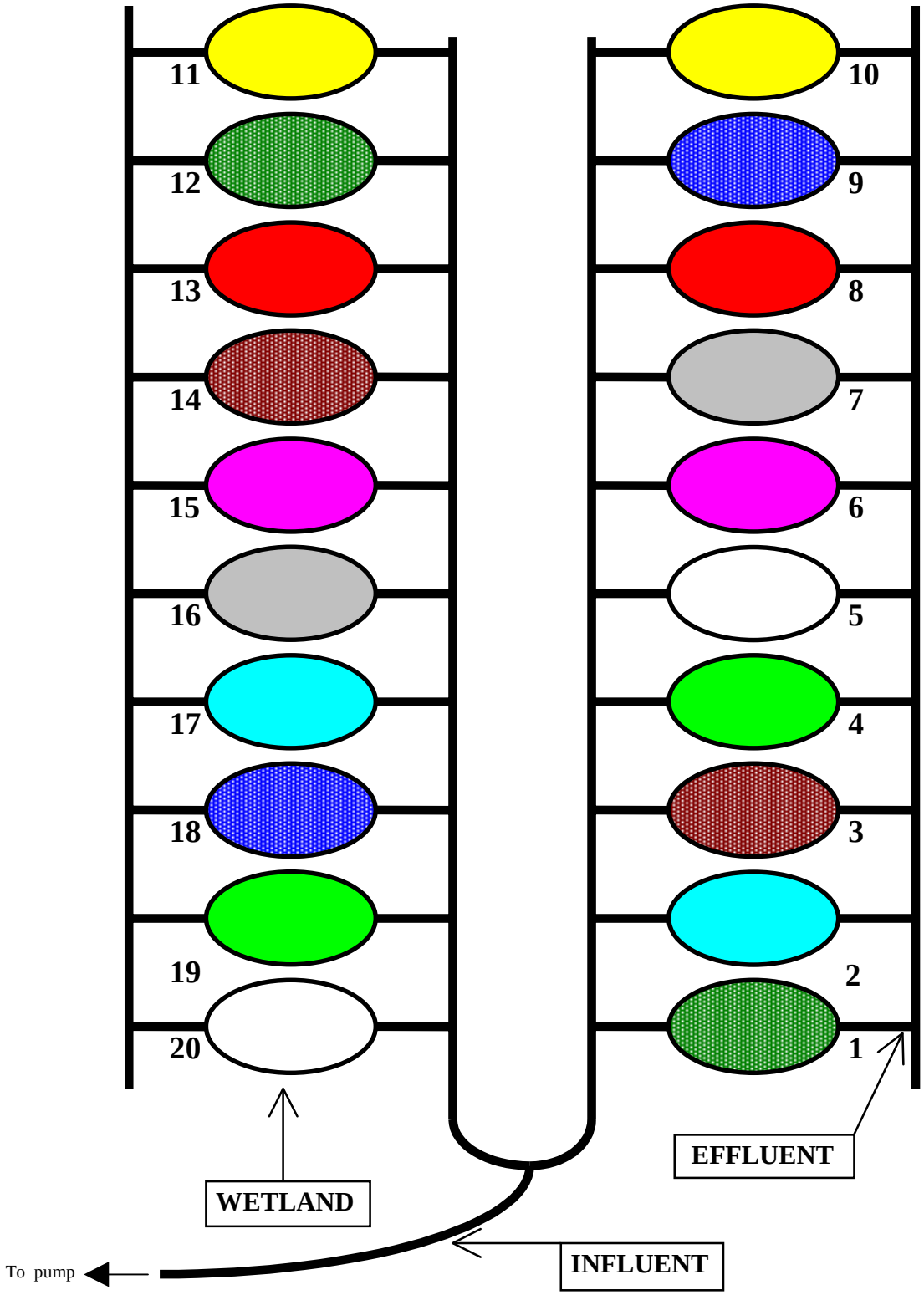

Figure 2.2.1. Schematic diagram of constructed wetland site located at the Morgantown, WV sewage treatment plant. See Table 2.2.1 for key to treatments and depths in this figure.

TABLE 2.2.1. Summary of treatments for constructed wetlands

Plant treatment	Wetland number[a]	Color
No plants	4s, 19s	green
No plants	7d, 16d	gray
Scirpus spp.	2s, 17s	blue
Scirpus spp.	3d, 14d	brown
Juncus spp.	8s, 13s	red
Juncus spp.	5d, 20d	white
Typha spp.	10s, 11s	yellow
Typha spp.	9d,18d	dark blue
Mixture	6s, 15s	pink
Mixture	1d, 12d	dark green

[a] Wetland cell number followed by a letter denotes depth of cell, (s)hallow or (d)eep

on B25 and B50 media, the resuscitative technique described by Munoa and Pares (35) was employed.

Wetland samples were diluted in 0.1% peptone, and 0.1 ml portions were spread plated onto RCA (Difco) and incubated at 35°C for 5 h under anaerobic conditions (GasPak, BBL). Twenty ml of modified RCA, now designated B25 or B50, was carefully overlaid onto the plates, and were then incubated for 4 days under the same conditions described above. The only modification to the Munoa et al. (35) procedure was to shorten the incubation from five to four days.

2.2.4 Fecal coliform enumeration. Fecal coliforms were recovered using either the standard membrane filtration technique described in *Standard Methods for the Examination of Water and Wastewater* (2) with 0.45 um filters placed on mFC agar or by appropriately diluting each sample followed by spread plating an aliquot onto mFC agar. These plates were incubated aerobically at 44.5°C for 24 h.

2.2.5 Enterococci enumeration. Enterococci were recovered either by the standard membrane filtration technique as described in *Standard Methods for the Examination of Water and Wastewater* (2) or by appropriately diluting each sample followed by spread plating an aliquot onto m-Enterococcus agar (Difco). These plates were incubated aerobically at 35°C for 48 h.

2.2.6 *Bifidobacterium* spp. presumptive identification. For the B25 and B50 media, Munoa et al. (35) reported a high degree of correlation between colonial morphology and type of bacteria enumerated. These criteria, as described below, were adopted in the current study to categorize the colonial types found on the samples from the constructed wetlands. White colonies with a diameter that clearly

exceeded 2 mm were always *Bifidobacterium* spp. All red colonies with a diameter less than 2 mm were formed by facultatively anaerobic Gram-positive cocci. Pink colonies were formed by cocci, bifidobacteria, and other rods. No Gram-negative bacteria were isolated on B25.

Resnick et al. (42) described bifidobacteria colonies on YN6 as presumptively positive if they were 1 to 2 mm in diameter, green (light-dark), circular, entire, convex or pulvinate, smooth, butyrous, and opaque and displaying typical bifidobacterial morphology upon Gram staining. These characteristics were used to distinguish bifidobacterial colonies on YN6 plates.

On all three bifidobacterial selective media, colony counts were categorized into the major colonial groups found on the media. For B25 and B50, these categories were, white, pink, and red. On YN6, these categories were small blue-green (circular), large blue-green (irregular), white, and filamentous.

2.2.7 Isolation of presumptive bifidobacterial colonies. For B25 and B50 plates, all presumptive bifidobacterial colonies were located between the agar layers. Isolating one particular colony necessitated extreme caution to prevent contamination of the isolate. One hundred µl sterile pipet tips were inverted and inserted into the agar with a circular twist until the top agar layer was penetrated. Then, using heat sterilized forceps, the plug was removed from the plate and placed into a screw-cap test tube containing 10 ml of RCM. These tubes were vortexed and incubated anaerobically (GasPak) at 35°C for 48 to 72 h. Thereafter, a 1 ml aliquot from each sample was diluted and spread plated onto B25. These plates were again incubated under the conditions described above. A colony was picked from these plates which

typified the characteristic bifidobacterial colonial morphology. This procedure was repeated two more times for each isolate to ensure a homogeneous sample. The resultant isolates were examined for their Gram reaction, motility, ability to grow aerobically, and carbohydrate utilization. This procedure is summarized in Figure 2.2.2.

2.2.8 Bifidobacterial enumeration media. Modified bifidobacteria enumeration media were used to test the feasibility of using bifidobacteria as fecal indicators. Modifications included lowering the pH, altering the carbon source, and/or the addition of selective agents (Table 2.2.2). These modified media were named bifidobacterial enumeration media (BEM). In addition, a similar resuscitative technique used by Munoa and Pares (35), whereby the samples were spread-plated on a non-selective basal medium and incubated for five hours and then overlaid with the selective agar, was employed to maximize bifidobacterial recovery. The challenge here was to remove possible extraneous carbon sources, such as starch, sodium acetate, and beef extract, as well as to use a more selective carbohydrate source. These modifications combined with the antibiotic regime from the B25 or B50 formulations, a pH indicator, such as phenol red, and/or propionic acid were used in an effort to increase differentiation and selectivity for bifidobacteria. All B25, B50, and BEM plates were overlaid with the respective basal formulations plus the antibiotics and selective agents, but not the pH indicator

2.2.9 Fermentation patterns of bifidobacteria isolates. Ten bifidobacteria isolates gathered from the constructed wetlands were examined for and differentiated on their carbohydrate fermentation patterns. Modified MRS broth reported by Roy et

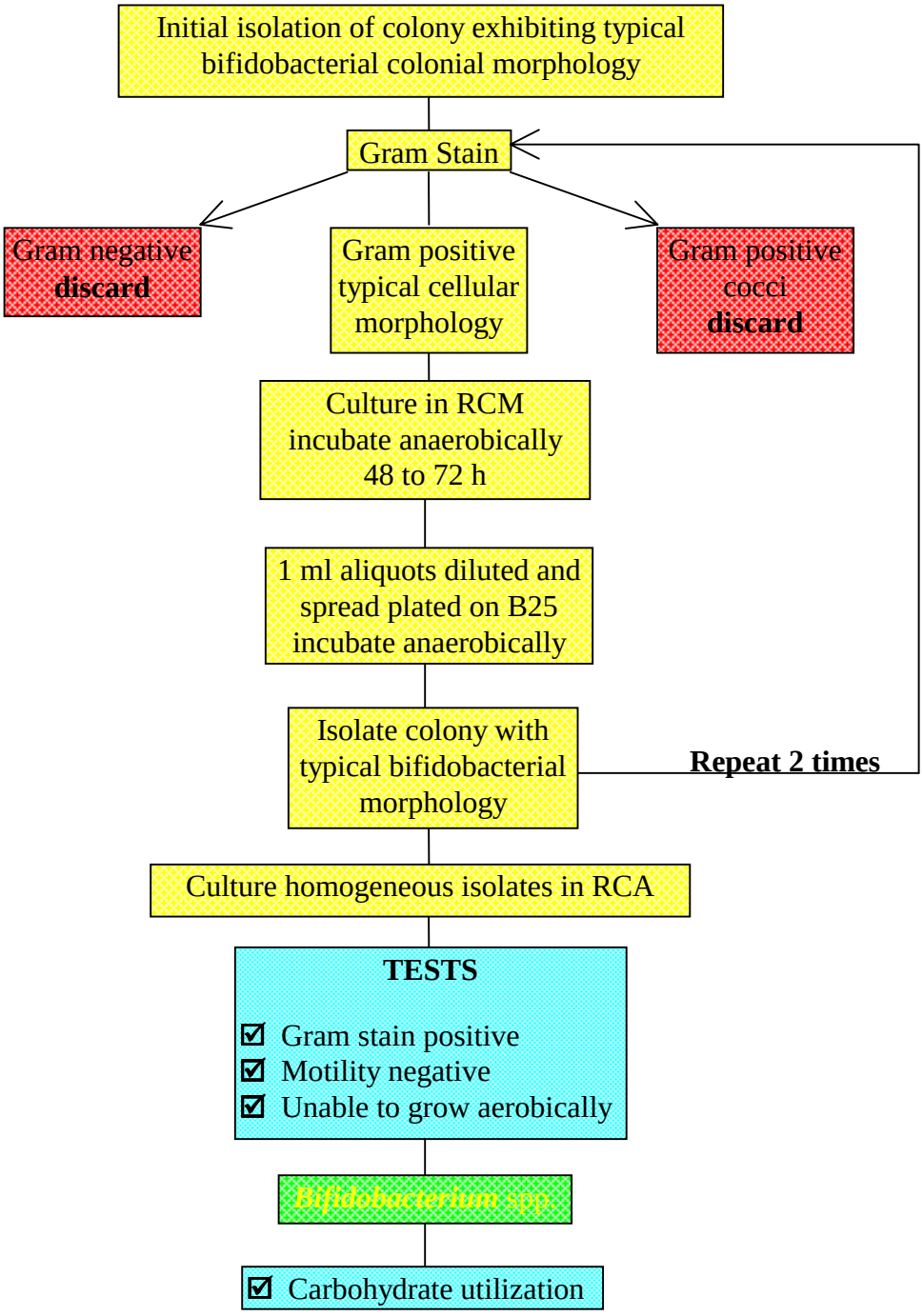

Figure 2.2.2. Identification of isolates as members of the genus *Bifidobacterium*.

Table 2.2.2a. Selective medium formulations with Reinforced Clostridial Agar as the basal component.

Compound	Medium[a]				
	B25	B50[b]	B25B	B50B[b]	B50B1[b]
Reinforced Clostridial Agar[c]	X	X	X	X	X
Bromocresol Green				X	X
Iodoacetic Acid	X	X	X	X	X
Kanamycin sulfate	X	X	X	X	X
Nalidixic Acid	X	X	X	X	X
Polymyxin B sulfate	X	X	X	X	X
Propionic Acid			X		X
Sodium Hydroxide (10 N)			X		X
2,3,4-triphenyltetrazolium chloride	X	X	X	X	X

[a] Agar overlay technique described by Munoa *et al*. (35) was used in all plating experiments
[b] B50, B50B, and B50B1 formulations contain double the concentration of iodoacetic acid
[c] Stock medium formulation contains tryptose, beef extract, yeast extract, dextrose, NaCl, starch, cysteine-HCl, sodium acetate, and agar

Table 2.2.2b. Basal and selective Bifidobacterial Enumeration media (BEM) formulations.

Compound	Medium[a]					
	BEM[b]	BEM1	BEM1B	BEM3	BEM6B	BEM6C
Tryptose	X	X	X	X	X	X
Yeast extract	X	X	X	X	X	X
Sodium chloride	X	X	X	X	X	X
Cysteine-HCl	X	X	X	X	X	X
Agar	X	X	X	X	X	X
Bromocresol green[c]			X			
Bromocresol purple[c]					X	
Dextrose				X		
Lactose		X	X		X	X
Phenol Red[c]						X
Nalidixic acid[d]		X	X	X	X	X
Polymixin B sulfate[d]		X	X	X	X	X
Kanamycin sulfate[d]		X	X	X	X	X
Iodoacetic acid[d]		X	X	X	X	X
2,3,4-triphenyltetrazolium chloride[d]		X	X	X	X	X

[a] Agar overlay technique described by Munoa *et al*. (35) was used in all plating experiments
[b] Basal BEM formulation is as follows (g/l): trypotse (10), yeast extract (3), NaCl (5), cysteine-HCl (0.5), and agar (15).
[c] pH indicators were included in the bottom layer only
[d] Selective agents were prepared as described by Munoa et al. (35) and included in the upper layer but not the lower layer of agar

al. (45) supplemented with 6 different sugars (Table 2.2.3) was used to observe these carbohydrate utilization patterns. Each isolate was inoculated into MRS broth supplemented with arabinose, cellobiose, lactose, mannose, ribose, or salicin. Inoculated tubes were incubated anaerobically in GasPak jars (BBL) at 35°C for 72 to 96 h.

Acid production without gas from carbohydrate fermentation was considered to be a positive reaction and was detected by a change in color of the phenol red indicator from red to yellow.

2.2.10 Statistical analysis. All statistical procedures were computed using SigmaStat (Jandel Scientific, San Rafael, CA). Natural logarithmic transformations of the observed counts were obtained.

Table 2.2.3. Composition of modified MRS broth

Compound	Concentration (g/l)
Proteose peptone no.3	10
Casamino acids	5
Yeast extract	10
Beef extract	1
Tween 80	1 ml
Ammonium acetate	2
$MgSO_4$	0.1
$MnSO_4 \bullet H_2O$	0.05
Na_2SO_4	2
KH_2PO_4	1.92
Na_2CO_3	0.2
$CaCl_2 \bullet 2H_2O$	0.1
L-cysteine-HCl	0.5
Phenol red	0.18
carbohydrate[a]	1.0%

[a] Carbohydrates used: arabinose, cellobiose, lactose, mannose, ribose, and salicin.

RESULTS

2.3.1 Enumeration of bifidobacteria. A preliminary sampling from a limited number of wetlands was gathered to test the effectiveness of the bifidobacterial enumeration procedure. This survey consisted of sampling the influent, cell 1, and cell 11. These two cells were chosen because cell 1 was closest to the influent pipe and cell 11 was the furthest from the influent pipe. The results of this sampling are summarized in Table 2.3.1. High numbers of bifidobacteria were encountered in these samples with no more than a 2 log difference between bifidobacteria and fecal coliforms in the influent. Numbers of bifidobacteria were greater than the numbers of enterococci found in all three samples. The dissolved oxygen level in cell 1 was twice that in cell 11, but this may be a function of the temperature and depth of the wetland (Table 2.3.1).

Due to the success of the preliminary sampling, more samples were collected the following month. This time one sample from each plant treatment, a total of 10 samples, and the influent were collected (Table 2.3.2.). With the exception of cell 8, bifidobacteria were detected in all cells and the influent.

Occurrences and comparisons of bifidobacteria (BIF), fecal coliforms (FC), and enterococci (ENT) densitites in constructed wetlands receiving primary treated sewage on (a) September 1994 and (b) October 1994 are shown in Figure 2.3.1. Bifidobacteria were enumerated on B50 (and also YN6 agar for the September sampling), fecal coliforms were enumerated on mFC agar, and enterococci were enumerated on m-Enterococcus agar. Although bifidobacteria occurred in primary

Table 2.3.1. Initial comparison of recoveries of bifidobacteria (BIF), fecal coliforms (FC), and enterococci (ENT) from constructed wetlands receiving primary treated sewage sampled on September 9, 1994

				CFU/mL		
Sample	pH	Temperature(°C)	D.O.(mg/L)	BIF[a]	FC	ENT
Influent	6.97	24.3	0.28	1.8×10^5	1.8×10^7	2.4×10^4
Cell 1	6.61	19.2	2.56	1.9×10^3	4.5×10^3	1.0×10^2
Cell 11	6.71	22.2	1.18	2.1×10^4	8.0×10^5	9.7×10^2

[a] Enumerated on B50.

Table 2.3.2. Comparison of recoveries of bifidobacteria (BIF), fecal coliforms (FC), and enterococci (ENT) from constructed wetlands receiving primary treated sewage sampled on October, 5, 1994

				CFU/mL		
Sample	pH	Temperature(°C)	D.O.(mg/L)	BIF[a]	FC	ENT
Influent	6.67	23.0	0.17	2.4×10^5	5.0×10^7	2.9×10^5
Cell 1	5.85	15.5	1.65	1.0×10^3	8.0×10^2	4.3×10^2
Cell 2	6.46	15.7	0.73	1.0×10^2	2.7×10^3	4.8×10^1
Cell 3	6.41	15.4	1.63	1.2×10^3	4.0×10^2	8.9×10^1
Cell 4	6.84	16.0	0.53	1.8×10^3	6.3×10^3	1.2×10^2
Cell 5	6.64	16.0	1.05	1.9×10^3	6.0×10^3	5.1×10^1
Cell 6	6.09	14.7	2.12	1.0×10^2	1.5×10^3	1.6×10^1
Cell 7	6.83	15.7	0.79	2.0×10^2	9.0×10^2	1.8×10^1
Cell 8	6.53	14.8	1.13	ND[b]	1.0×10^2	<1
Cell 9	6.39	15.7	2.14	1.0×10^3	6.0×10^2	5.0×10^0
Cell 11	6.23	16.2	0.78	2.0×10^2	1.4×10^3	4.4×10^1
All Cells[c]	6.42	15.6	1.13	4.9×10^2	1.1×10^3	4.5×10^1

[a] Enumerated on B50.
[b] ND, not detected.
[c] Geometric mean.

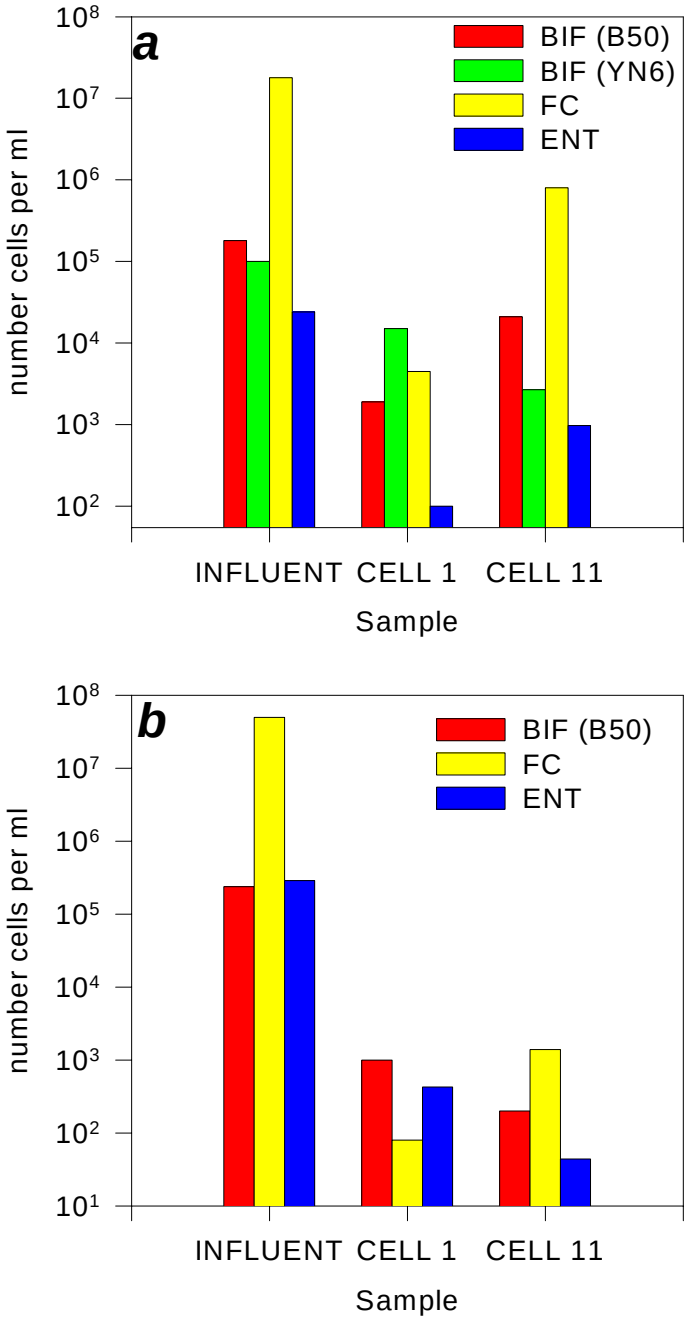

Figure 2.3.1. Occurrences and comparisons of bifidobacteria (BIF), fecal coliforms (FC), and enterococci (ENT) densities in constructed wetlands receiving primary treated sewage on (a) September 1994 and (b) October 1994

treated sewage approximately 100-fold less than fecal coliforms, they exhibited a 100- to 1000-fold less reduction than the fecal coliforms after passage through the wetland.

Not shown in Tables 2.3.1 and 2.3.2 was the performance of YN6 agar. YN6 agar was observed to be inadequate for enumerating bifidobacteria from primary treated sewage or from any of the wetlands. With the original samples in September, YN6 enumerated bifidobacteria in densities similar to those obtained on B50. However, when YN6 was used in a second sampling date in September and one in October, no colonies grew on this medium. In addition, by the time the 4-day incubation was finished, many of the YN6 plates were overgrown with fungus-like organisms notably at the lower dilutions (not shown). This inconsistency in the performance of YN6 is unacceptable in its use to enumerate bifidobacteria – especially in a laboratory testing possible fecal contamination of a potable water supply.

The samples taken in September and October 1994 demonstrated that bifidobacteria can be observed in the constructed wetlands and in primary treated sewage. Wetland cells 1 and 11 and the primary treated sewage were then chosen to be monitored over the next 4 months to see how the densities of bifidobacteria, fecal coliforms, and enterococci fluctuated with the natural changes in temperature, moisture, light, etc. The occurrence of bifidobacteria, fecal coliforms, and enterococci in primary treated sewage over time is shown in Figure 2.3.2. Fecal coliforms maintained a higher population than either the bifidobacteria or enterococci during the course of the experiment. The bifidobacteria exhibited less fluctuation in

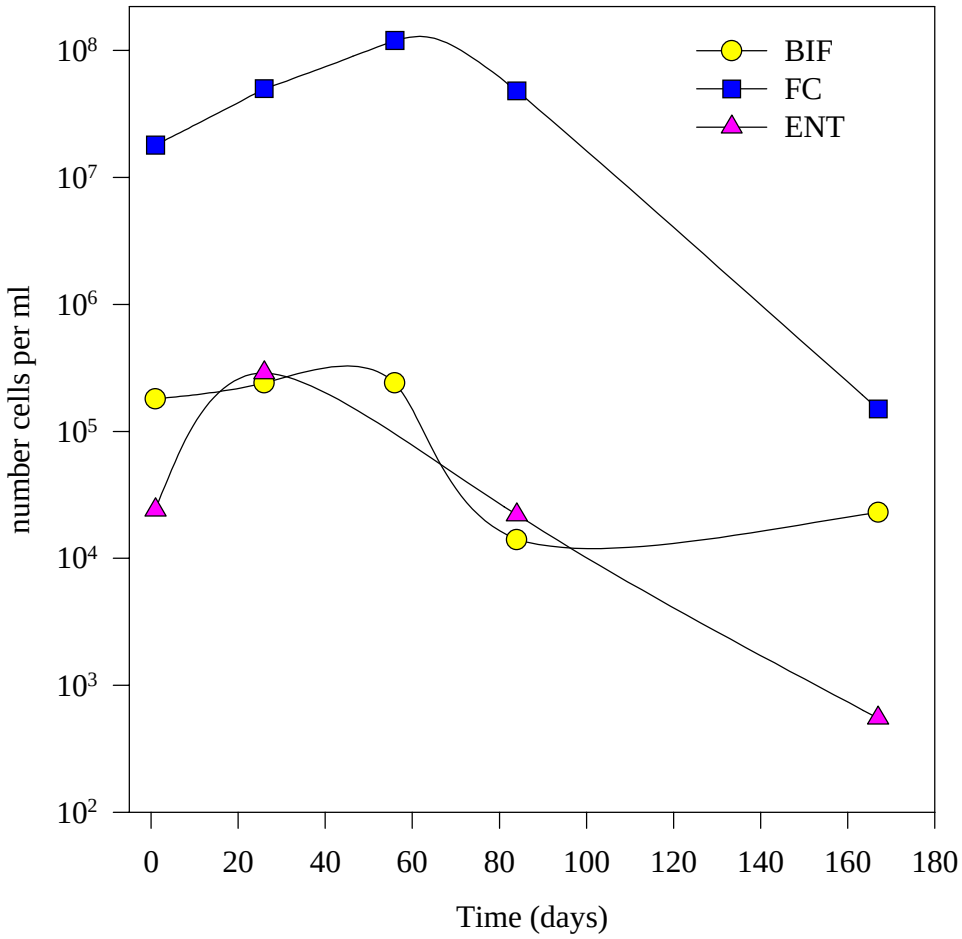

Figure 2.3.2. Occurrence of bifidobacteria (BIF), fecal coliforms (FC), and enterococci (ENT) in primary treated sewage beginning September, 1994. Bifidobacteria were enumerated on B50, FC were enumerated on mFC agar, and ENT were enumerated on mEntercoccus agar.

their density over time than the fecal coliforms – always within approximately one order of magnitude for all sampling dates. This corroborates the data from the laboratory microcosm experiments at 13°C in Chapter 1. Bifidobacteria in those microcosms exhibited a longer survival periods and there were cold temperatures occurring between the 4th (December) and 5th (March) sampling times in the wetlands.

The occurrence of bifidobacteria, fecal coliforms, and enterococci in constructed wetland cell 1 is shown in Figure 2.3.3. The density of fecal coliforms was reduced by the wetland by approximately 3.5 orders of magnitude on all sample dates. Bifidobacteria were reduced by approximately 2 logs and again exhibited less fluctuation in their density over time than the fecal coliforms. Figure 2.3.4 shows the occurrence of bifidobacteria, fecal coliforms, and enterococci in wetland cell 11. Initial reductions of all three indicator organisms were approximately 1 order of magnitude. However, by the third sample date (November), fecal coliforms were reduced by 6 orders of magnitude, bifidobacteria by approximately 3 orders of magnitude, and enterococci were on the verge of becoming non-detectable. These quite large reductions could be attributable to brief periods of cold weather causing the wetland cells to freeze. When frozen, the influent was turned off to prevent the primary treated sewage from spilling over the rim of the wetland cell.

2.3.2 *Bifidobacterium* enumeration media. Results from the initial samples showed that YN6 agar was an ineffective medium due to its lack of selectivity. On some sampling dates YN6 plates exhibited overgrowth from filamentous organisms, while on other dates no growth was apparent on any YN6 plates even though growth

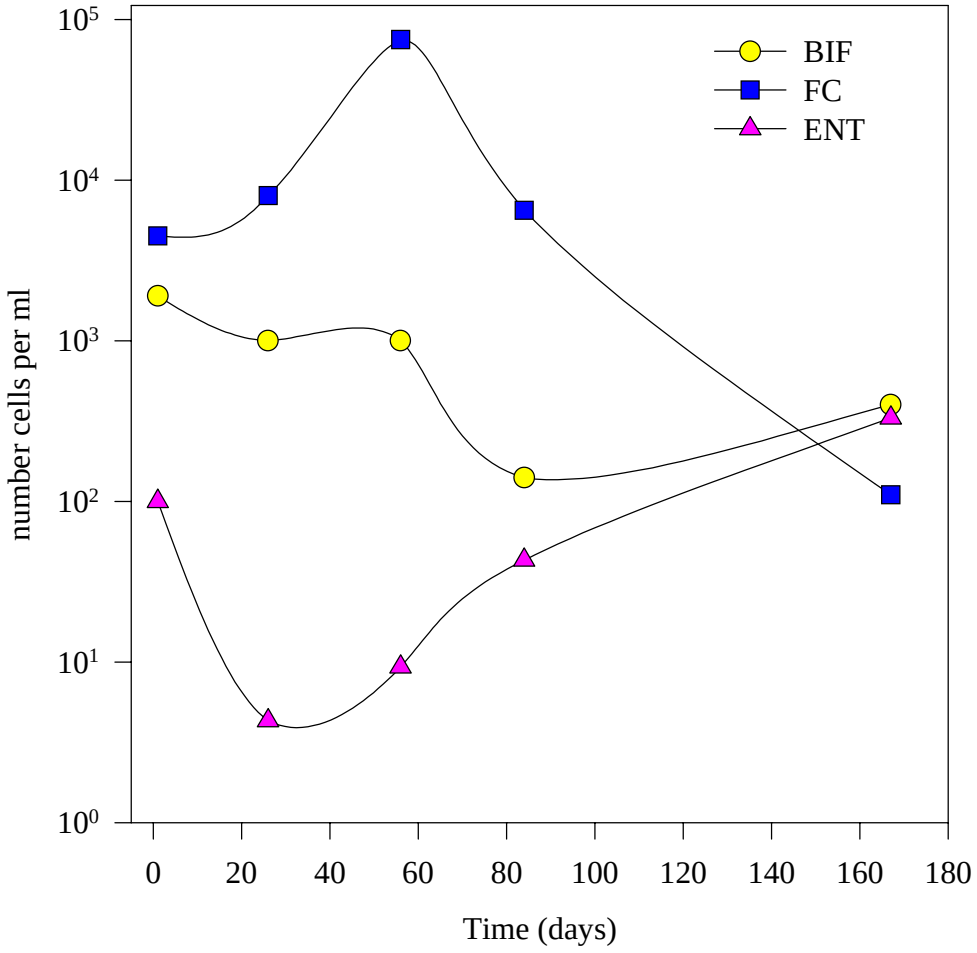

Figure 2.3.3. Occurrence of bifidobacteria (BIF), fecal coliforms (FC), and enterococci (ENT) in constructed wetland cell 1 beginning September, 1994. Bifidobacteria were enumerated on B50, FC were enumerated on mFC agar, and ENT were enumerated on mEnterococcus agar.

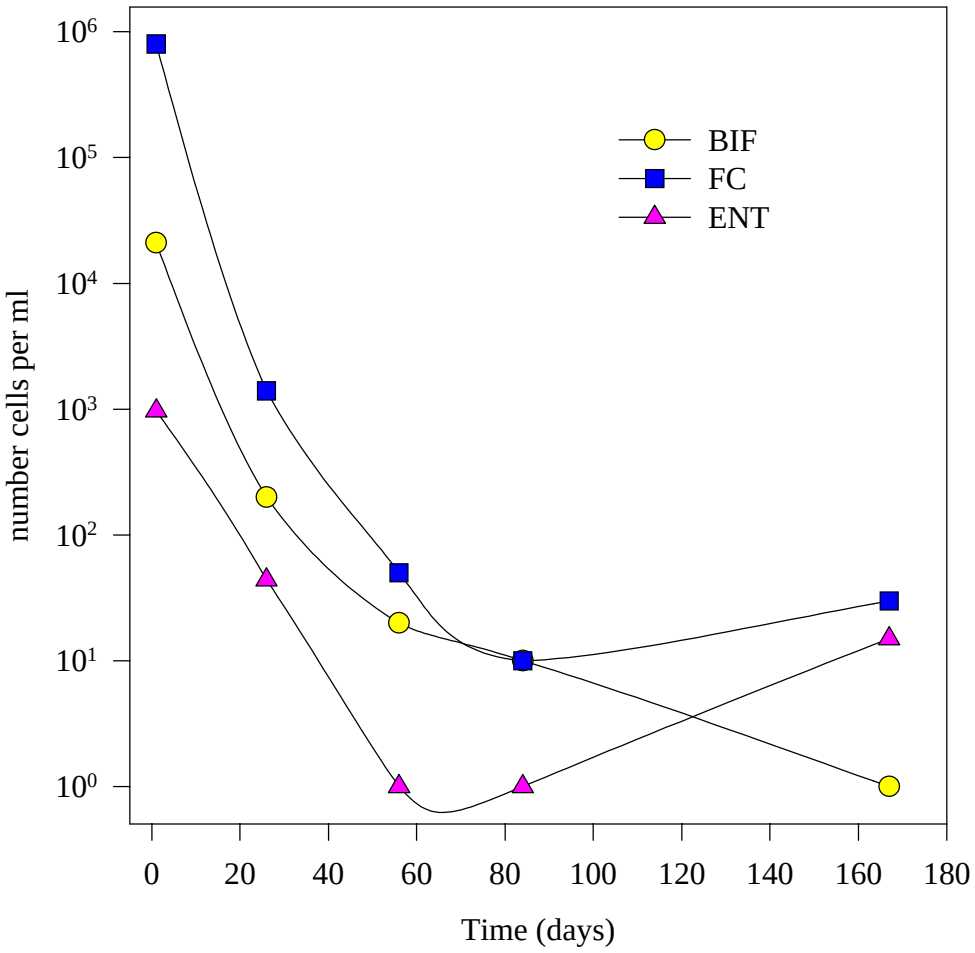

Figure 2.3.4. Occurrence of bifidobacteria (BIF), fecal coliforms (FC), and enterococci (ENT) in constructed wetland cell 11 beginning September, Bifidobacteria were enumerated on B50, FC were enumerated on mFC agar, and ENT were enumerated on mEnteroccus agar.

was observed on other bifidobacterial selective media at the same dilutions. Efforts were made to slightly modify the formulation and preparation of the YN6 medium, but the enumeration problems continued. Because of this lack of selectivity, reproducibility and reliability, the use of YN6 was discontinued in this study.

It became clear after a few samples that the present isolation media needed modification in order to more effectively enumerate the bifidobacteria. Therefore, an effort was made to modify these media by using a lower pH, changing the main carbon source, eliminating extraneous carbon sources, and adding additional selective agents, such as propionic acid. These modified media were named Bifidobacteria Enumeration Media (BEM) and numbered sequentially with each modification (Table 2.2.2). All modified media were inoculated concurrently with B25 or B50 for comparison on subsequent samplings. Promising media were those that inhibited more effectively by percentage the large number of background colonies that appeared on BIM25 and BIM50 as well as differentiated more clearly between bifidobacterial and nonbifidobacterial colonies.

Both B25 and B50 media were semi-sensitive in enumerating bifidobacteria. B50 was used in conjunction with B25 to observe the effects of increasing the selectivity of the medium on bifidobacteria recovered from aquatic samples. This effect is shown in Figures 2.3.5 and 2.3.6 which denote the relative numbers of colonial morphological types for constructed wetland cell 1 and the primary treated sewage, respectively. Since the formulation of the media differed only in the concentration of iodoacetic acid, the expected colonial types were similar. While bifidobacteria represented 21.4 percent of the total bacteria enumerated on B25 agar

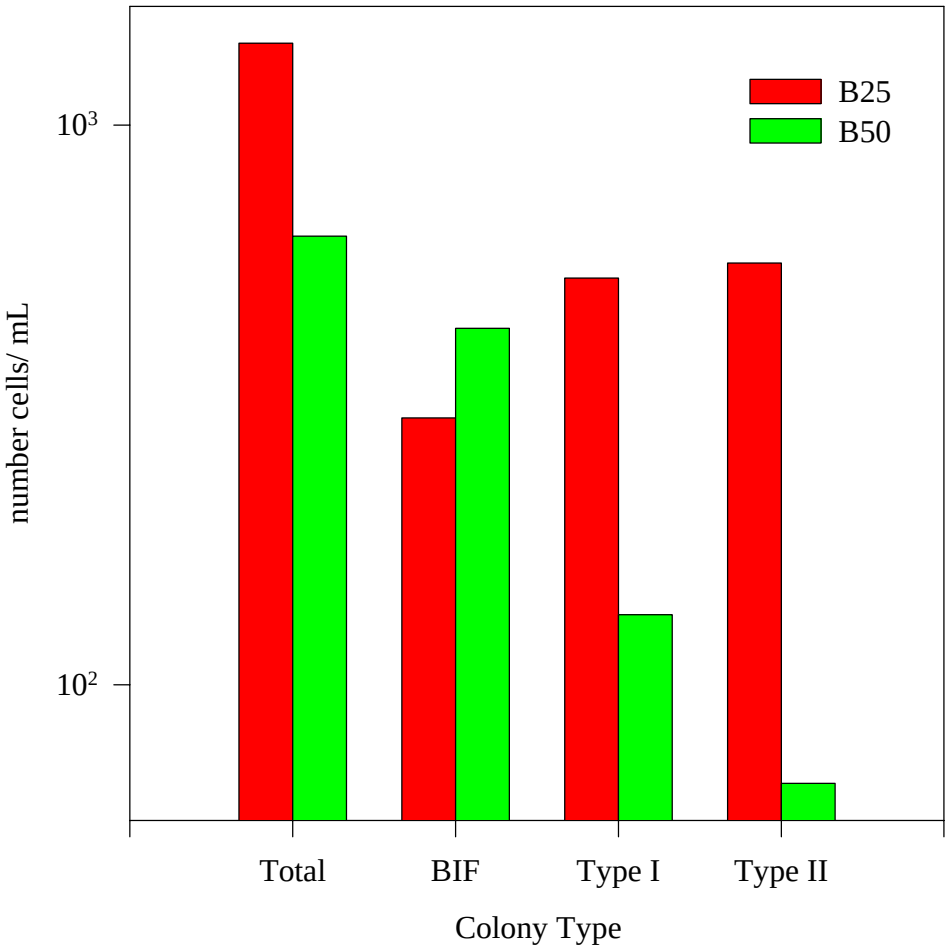

Figure 2.3.5. Occurrence of major colonial morphology categories on B25 and B50 agar from constructed wetland cell 1 sampled on September 9, 1994. The BIF designation covers white colonies which indicated bifidobacteria, Type I were red colonies which indicated Gram-positive anaerobic cocci, and Type II were pink colonies which covers cocci, bifidobacteria, and other rods.

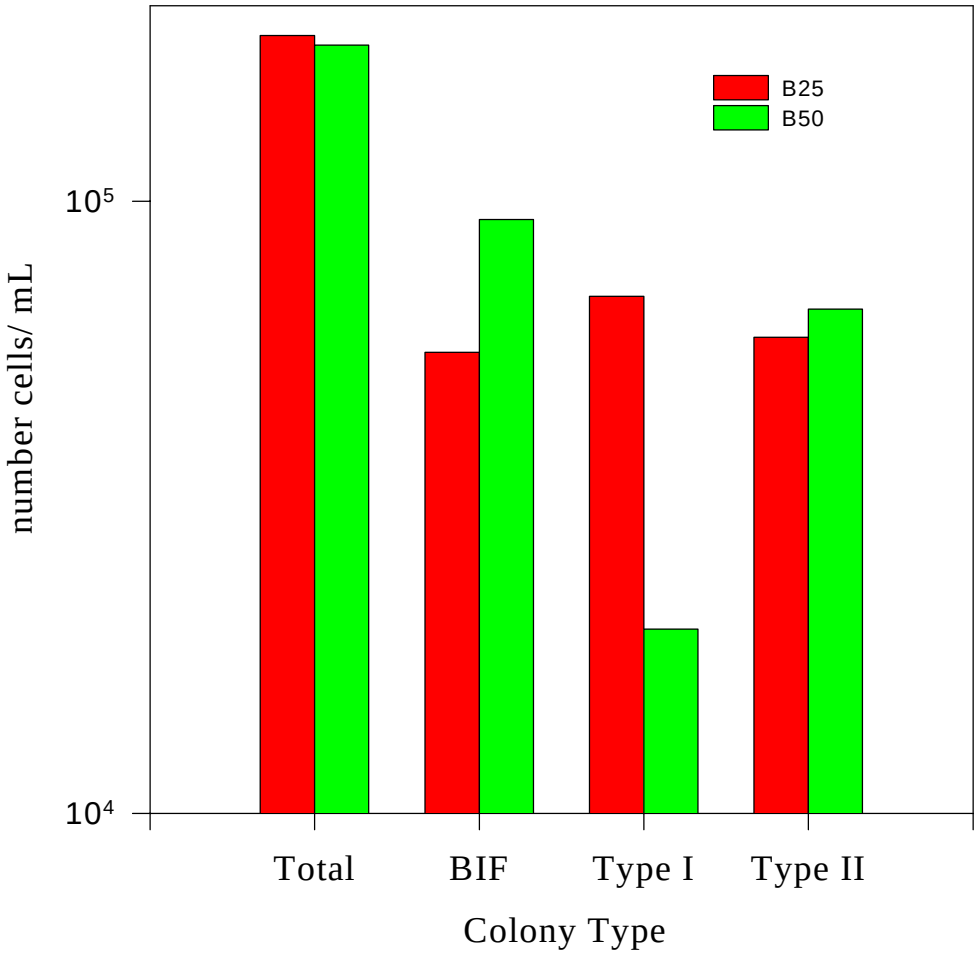

Figure 2.3.6. Occurrence of major colonial morphology categories on B25 and B50 agar from primary treated sewage sampled on September 9, 1994. The BIF designation covers white colonies which indicated bifidobacteria, Type I were red colonies which indicated Gram-positive anaerobic cocci, and Type II were pink colonies which covers cocci, bifidobacteria, and other rods.

from cell 1 (Figure 2.3.5), they comprised 68.4 percent of the total number of bacteria enumerated on B50. The red colonial type constituted 38.1 percent of the total number of cells on B25 while that number dropped to 21.1 percent on B50. The pink colonial type, which could have included some bifidobacterial colonies, dropped from 40.5 percent of the total on B25 to 10.5 percent when enumerated on B50.

Similar results were seen in the primary treated sewage (Figure 2.3.6). Bifidobacteria comprised 30.4 percent of the total when enumerated on B25, while they increased to 51.9 percent of the total when enumerated on B50. The occurrence of the red colonial type dropped from 37.5 percent of the total when enumerated on B25 to 11.1 percent of the total when enumerated on B50. While the percentage of pink colonies increased from 32.1 percent on B25 to 37.0 percent on B50, a possible explanation could be that the increase was actually bifidobacterial colonies exhibiting the pink colonial morphology. Munoa et al. (35) observed that a portion of the pink colonial type were confirmed as bifidobacteria.

The occurrence of bifidobacteria on B50 and BEM3 media in (a) primary treated sewage and (b) constructed wetland cell 11 sampled on September 23, 1994 is shown in Figure 2.3.7. Major colonial types on B50 consisted of white colonies representing bifidobacteria, red colonies representing Gram-positive cocci (type 1), and pink colonies that consisted of cocci, bifidobacteria and other rods (type 2). BEM3, lacking beef extract, appeared more pale in color than B50 and exhibited the same main colonial morphologies as B50. The paleness of BEM3 contributed to an increased difficulty in distinguishing bifidobacterial colonies as they appeared white

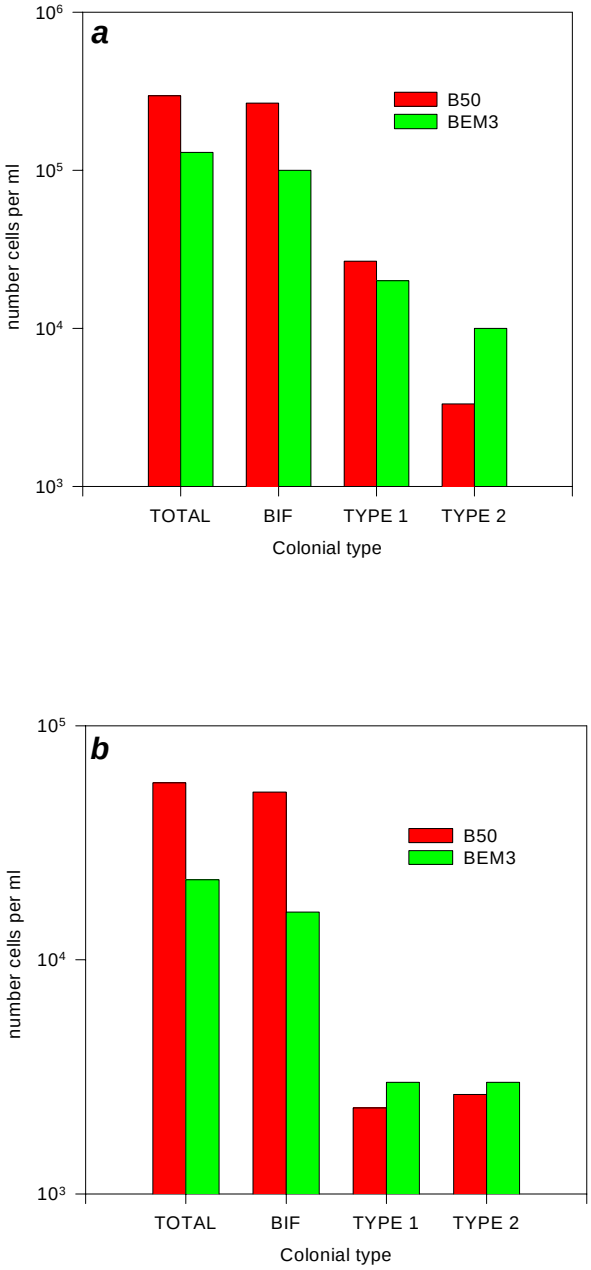

Figure 2.3.7. The occurrence of bifidobacteria on B50 and BEM3 media in (a) primary treated sewage and (b) constructed wetland cell 11 sampled on September 23, 1994

underneath a white agar overlay. B50 adequately enumerated bifidobacteria from both primary treated sewage and cell 11. Bifidobacteria comprised 89.9% of the total CFU on the B50 plates in the primary treated sewage and 91.2% of the total CFU on the B50 plates in cell 11. BEM3, which contains dextrose as the major carbohydrate source, did not inhibit non-bifidobacteria as effectively as B50 nor did it recover as many bifidobacteria as were observed on B50.

Figure 2.3.8 shows a comparison of various bifidobacteria enumeration media for monitoring the occurrence of bifidobacteria in primary treated sewage on December 7, 1994. On all media with the exception of B50b1, which contained propionic acid as an additional selective agent, there were three main types of colonies observed. It appeared that the addition of propionic acid (5ml/l) – the same amount used by Beerens (7) -- further reduced the number of Gram positive cocci (Type 1 in figure) and effectively inhibited the occurrence of the Type 2 category documented by Munoa et al. (35) observed. However, the total number of CFU observed on B50b1 was approximately one-third the total observed on B50. The total number of CFU on B50b1 was less than the number of presumptive bifidobacteria on B50. Therefore, B50b1 was considered too inhibitory to the bifidobacterial population to be used as a quantitative medium. BEM1 effectively enumerated bifidobacteria in comparable numbers to B50 with the additional advantage of easier differentiation of bifidobacterial CFU. This may explain the slight increase in type 1 CFU counts for the BEM1 compared to the B50 plates. BEM1 does not contain the extraneous carbon sources found in RCA which is the basal medium used in the formulation of B50. In addition, BEM1 contains lactose as a main carbohydrate

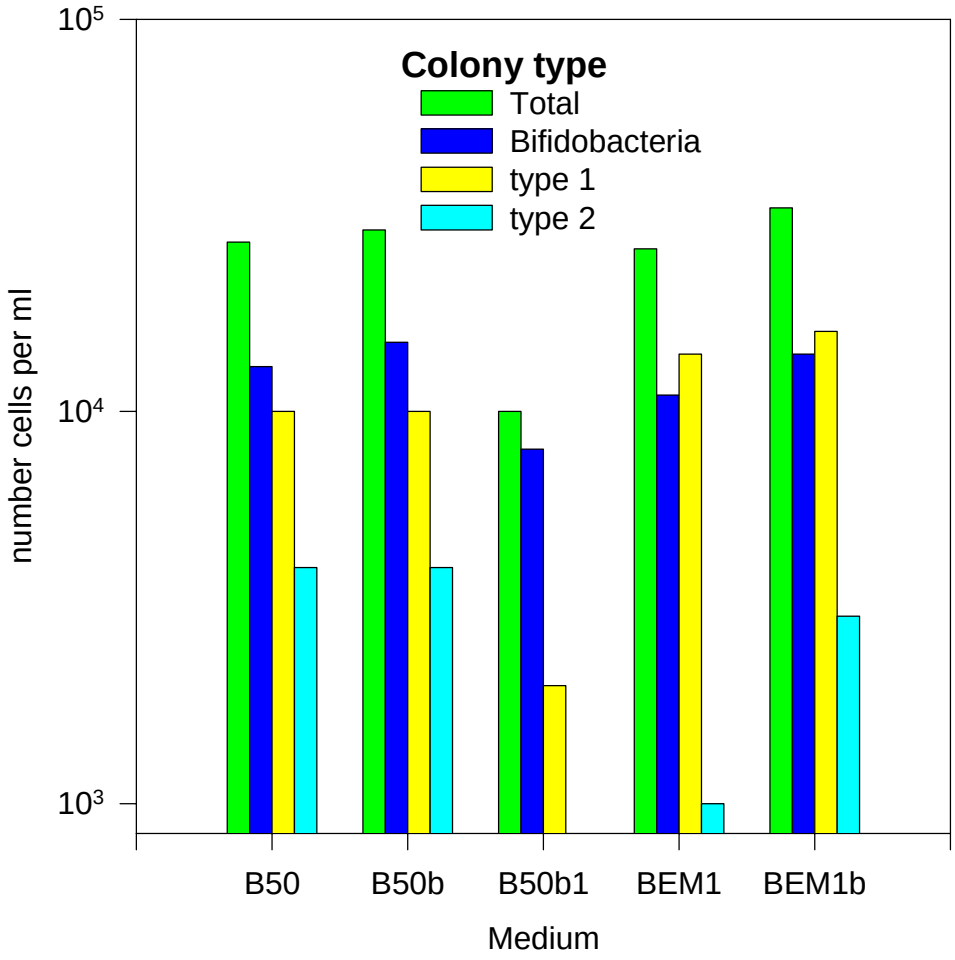

Figure 2.3.8. Comparison of various bifidobacteria enumeration media for monitoring the occurrence of bifidobacteria in primary treated sewage on December 7, 1994.

source as opposed to dextrose in the B50 formulation. BEM1b, which contains bromocresol green as a pH indicator, increased the ability to differentiate bifidobacteria CFU over BEM1. Any growth of bifidobacteria on these plates caused a ring of yellow which increased as the colonies grew due to the production of acid by the bifidobacteria. Although it was assumed that other types of bacteria growing on the plates could produce acid and therefore zones of yellow, the color change facilitated bifidobacteria colony recognition in conjunction with the other colonial morphology parameters used. The occurrence of bifidobacteria on B50, BEM6B, and BEM6C media in constructed wetland cell 11 sampled on July 7, 1995 is shown in Figure 2.3.9. White colonies represent bifidobacteria, type 1 colonies denote small red colonies, and type 2 colonies denote pink colonies. Although B50 enumerated bifidobacteria from the wetland, its inability to effectively inhibit non-bifidobacteria and differentiate bifidobacteria makes this medium inefficient to regularly monitor bifidobacteria from even moderate sample numbers. BEM6B, which contains lactose as the major carbohydrate source and bromocresol purple as a pH indicator, did not effectively enumerate or differentiate bifidobacteria. BEM6C differed from BEM6B only in that phenol red was used as the pH indicator. Bifidobacteria comprised 28.4% of the total CFU observed on the BEM6C plates compared to 10.8% of the total observed on the BEM6B plates. Phenol red appeared to be less inhibitory than bromocresol purple to the bifidobacteria population. However, BEM6C was not sufficiently selective as 71.6% of the total CFU were considered non-bifidobacteria.

2.3.3 Bifidobacterial fermentation patterns. Ten bifidobacteria isolates gathered from the constructed wetlands and the primary treated sewage were

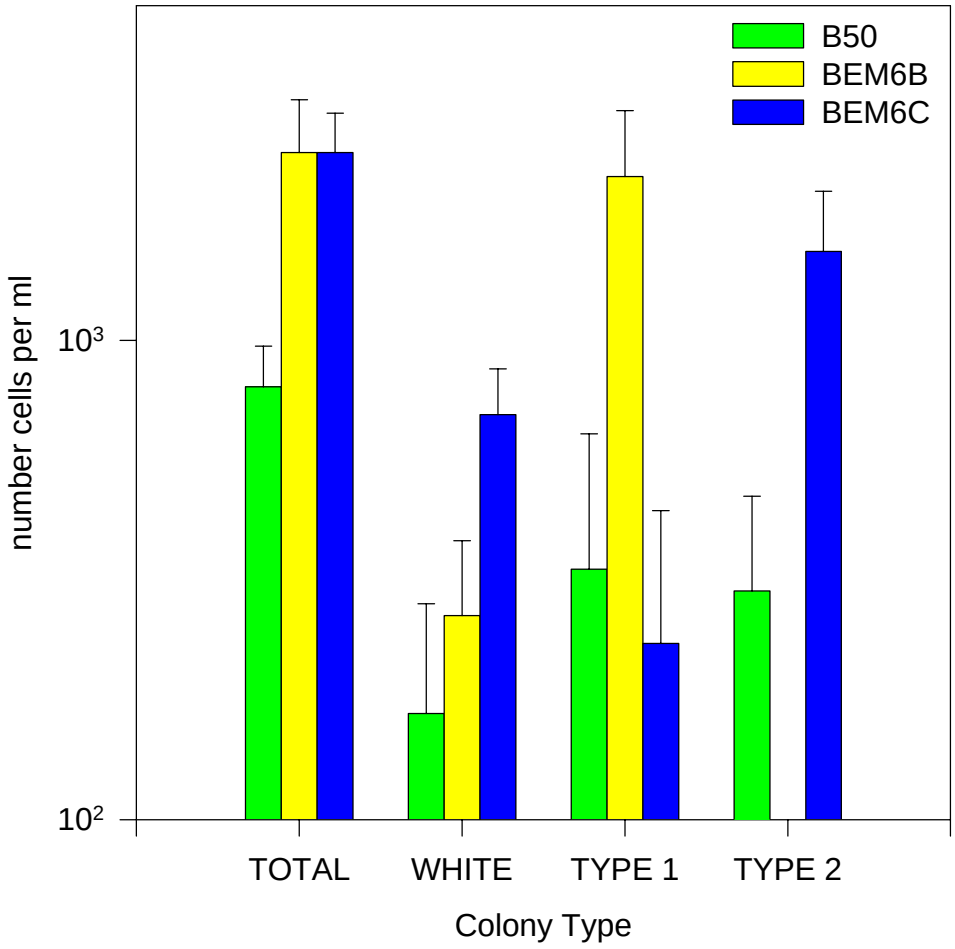

Figure 2.3.9. The occurrence of bifidobacteria on B50, BEM6B, and BEM6C media in constructed wetland cell 11 sampled on July 7, 1995. . Error bars indicate the standard deviation.

examined for and differentiated on their carbohydrate fermentation patterns (Table 2.3.3). The results of the fermentation assay is shown in Table 2.3.4. Lactose was used as a positive control. Arabinose, salicin, mannose, ribose, and cellobiose differentiated the isolates into groups based on the scheme by Roy et al. (45). According to this scheme, bifidobacteria can be differentiated into two main groups based on their ability to ferment arabinose. All 10 environmental isolates examined were able to ferment arabinose. Furthermore, the environmental isolates all fell into two subgroups based on their ability to ferment salicin; salicin-negative (sub-cluster B1) and salicin-positive (cluster B2) groups. Some discrepancies were observed, but this can be attributed to stock ATCC strains being used in the study by Roy et al. (45) while this study used environmental isolates from the constructed wetlands. For example, isolates 4, 5, and 7 were keyed out to *Bifidobacterium pseudocatenulatum*. However, these isolates also fermented cellobiose, which is inconsistant with the fermentation pattern for that species according to Roy et al. (45). A more accurate species differentiation could be made if, in addition to the stock strains, known environmental isolates would be included in future studies. All isolates examined fell into groups that are of human origin. Suprisingly, not one of the isolates showed the fermentation characteristics of *B. bifidum*, one of the most ubiquitous species of this genus.

Table 2.3.3. Source and isolation medium of each environmental isolate used in the differentiation assay.

Isolate	Source[a]	Isolation medium[b]
1	8	BEM1 + PA
2	Inf	B25
3	1	BEM1 + PA
4	1	BEM1 + PA
5	8	BEM1 + PA
6	1	BEM1 + PA
7	1	BEM1 + PA
8	1	BEM1 + PA
9	1	BEM1 + PA
10	3	BEM1b

[a] Number indicates wetland cell number, inf indicates influent.
[b] See Table 2.2.2 for medium recipe, PA = propionic acid.

Table 2.3.4. Carbohydrate fermentation patterns of the 10 environmental bifidobacterial isolates.

	Carbohydrate						
Isolate	Lactose	Arabinose	Salicin	Mannose	Ribose	Cellobiose	Species
1	+	+	-	+	+	-	*longum* or *infantis*
2	+	+	-	+	+	-	*longum* or *infantis*
3	+	+	-	+	+	-	*longum* or *infantis*
4	+	+	+	+	+	+	*pseudocatenulatum*
5	+	+	+	+	+	+	*pseudocatenulatum*
6	+	+	+	+	+	-	*breve, infantis* or *pseudocatenulatum*
7	+	+	+	+	+	+	*pseudocatenulatum*
8	+	+	+	+	+	-	*breve, infantis* or *pseudocatenulatum*
9	+	+	-	+	+	-	*longum* or *infantis*
10	+	+	-	+	+	-	*longum* or *infantis*

DISCUSSION

Groundwater supplies 95 percent of the rural homes in the US and 97 percent of the rural homes on private supplies in West Virginia (1, 11). Unfortunately, the trend of groundwater pollution is increasing, and the most common water quality problem in rural water supplies is from bacterial contamination from septic tanks (54,55). Small constructed wetlands can be used as alternatives to traditional septic systems especially in situations where septic systems are rendered ineffective (19,24,58). The wetland acts to remove pathogens either through filtration or by retention in the wetland matrix.

The purpose of this study was to monitor the fate of bifidobacteria and traditional indicator bacteria in constructed wetlands receiving primary treated sewage. Bifidobacteria, a proposed indicator group, were monitored in an effort to describe their behavior in an external aquatic environment. As discussed in Chapter 1, bifidobacteria are exclusively of fecal origin, excreted in very high densities, do not exhibit aftergrowth outside the host, and some species are exclusive to humans. Detection of bifidobacteria in the constructed wetlands would indicate the presence of fecal material.

Two of the specific objectives of this study were to examine the occurrence of bifidobacteria in constructed wetlands receiving primary treated sewage and to observe the relative survival of bifidobacteria versus traditional indicator bacteria in the wetlands. Initial samples gathered from the wetlands and the primary treated

sewage demonstrated that bifidobacteria can be enumerated from these systems (Tables 2.3.1 and 2.3.2). The bifidobacterial population of the influent from the September sampling was approximately 2 X 10^5 CFU/ml and was reduced 90 to 99% after treatment in the constructed wetlands. This reduction may be primarily due to the increased level of dissolved oxygen in the wetlands compared to the influent. While densities of bifidobacteria averaged approximately 2 logs less than fecal coliforms in the primary treated sewage, their populations were similar to fecal coliforms after retention in the wetland. The advantage here would be that with a fermentation test, the presence of bifidobacteria could be directly linked to human fecal material. The main disadvantage would be the time involved to complete the presumptive and confirmation test. With a minimum of 4 days of incubation at each step, it would take about 2 weeks from the time of sampling until the confirmation results were available for each sample. The population of bifidobacteria in the wetlands exhibited less fluctuation over time than the fecal coliforms or enterococci. In both the primary treated sewage and wetland cell 1 sample, populations of bifidobacteria fluctuated by approximately 1 order of magnitude over the time period they were monitored. Even in the wetland 11 samples, bifidobacteria fluctuated over 4 orders of magnitude, which was still less than the fluctuation of fecal coliforms over the same period.

The second specific objective of this study was to evaluate published selective media for their sensitivity and selectivity for the recovery of bifidobacteria from the wetlands and the primary treated sewage. One major problem encountered in this part of the study was the ineffectiveness of YN6 as a reliable selective medium. YN6

exhibited a lack of selectivity for bifidobacteria from contaminated water samples; overgrowth by filamentous organisms were a problem on most of the sample dates YN6 was used, while periodically no growth at all was observed on the YN6 plates even though bifidobacterial densities on other selective media were in the 10^4 to 10^5 cells per ml range. The use of YN6 was discontinued about midway through the course of this study due to the reliability and reproducibility issues. It is this author's opinion that the YN6 formulation published by Resnick and Levin (42) should not be used to reliably enumerate bifidobacteria from aquatic samples.

B25 and B50 selective media enumerated bifidobacteria from the wetlands and primary treated sewage. The main problem with these media is their lack of selectivity. Approximately 70 to 80 percent of the cells enumerated on the B25 plates and approximately 30 to 50 percent of the cells enumerated on B50 were not considered even presumptive bifidobacterial colonies. At higher dilutions, overgrowth quickly appeared on these plates. Keep in mind as well that in the microcosm experiments discussed in Chapter 1, the use of B25 or B50 to enumerate bifidobacteria caused a significant underestimation of their density even at lower temperatures, so the true density of bifidobacteria in the wetlands may have been as much as an order of magnitude greater.

While not an original objective of this study, modification of published bifidobacterial selective media was performed in an attempt to improve the ability to enumerate and identify bifidobacteria from the wetland samples. The disadvantages of published selective media for the enumeration of bifidobacteria, namely the lack of ease and extensive amount of time involved in confirmation of presumptive colonies,

prevent the use of bifidobacteria as an indicator organism on a routine basis. The most effective system of microbiological monitoring of water sources requires simple, rapid, and relatively inexpensive tests to determine the presence of indicator bacteria on a routine basis (48). The lengthy procedures required for the preparation of media, incubation of plates, and confirmation of colonies give rise to high costs related to man-hours and materials and further compound the use of bifidobacteria as indicator organisms. The modifications were performed to simplify recognition of bifidobacteria colonies as well as increase recovery of sub-lethally injured cells. Increasing the ability to differentiate bifidobacterial colonies – at least in the presumptive stage – would decrease the time and materials needed in the confirmation process.

At first, the modifications consisted of additions to the B25 and B50 formulations found in the literature (35). These modifications included the addition of bromocresol green as a pH indicator and propionic acid as a selective agent. Bifidobacteria ferment dextrose to acetate and lactate, so a light yellow zone in the medium would surround a bifidobacterial colony. While other bacteria were assumed to be able to ferment the dextrose in the medium, the yellow zone combined with the distinctive colonial morphology would enhance the recognition of bifidobacteria. Propionic acid was included since it has been shown to inhibit the growth of members of the family *Enterobacteriaceae*, strains of *Enterococcus*, *Staphylococcus*, and *Micrococcus* spp., and other Gram-positive bacteria while most strains of bifidobacteria have been shown to grow on it to the same degree as a non-selective medium (6,7). Five ml/l of propionic acid was added to the B25 and B50

formulations and then adjusted to a pH of 5.0 with approximately 3 ml of 10 N sodium hydroxide.

A comparison of these modifications is shown in Figure 2.3.8. A slight increase in the numbers of bifidobacteria was observed on the B50b plates, but this can be attributed to higher overall numbers of bacteria since the numbers of type 1 and type 2 colonies were constant. Type 2 colonies were absent on B50b1 medium, but the overall numbers of bacteria enumerated was smaller than the number of bifidobacteria enumerated on either B50 or B50b. While it was stated in the Results section of this chapter that B50b1 was considered too inhibitory to be used as a quantitative medium, it should be noted here that the bifidobacterial colonies were quite easily differentiated from the other colonies and readily countable. As the main aim of the medium modifications was to simplify recognition of bifidobacterial colonies, the use of B50b1 would seem preferable to B50 for enumeration of bifidobacteria from a constructed wetland. One must keep in mind, however that given the performance of the published selective media compared to the non-selective medium in the controlled microcosm studies discussed in Chapter 1, use of B50b1 will result in an underestimation of a log or more of the true bifidobacterial population.

It was this problem of inhibition that prompted more extensive modifications involving the stock RCM formulations used as a basis for the B25 and B50 selective. Any possible extraneous carbon sources (i.e., beef extract, starch, sodium acetate, and dextrose) were removed so that the new basal medium, now called BEM for bifidobacterial enumeration medium, contained just tryptose, yeast extract, sodium

chloride, cysteine hydrochloride, and agar in the same concentrations as in the RCM medium. Since bifidobacteria are enteric organisms, lactose was chosen as the main carbon source to be added back into the basal BEM medium in the hope that the lactose would be more selective than the dextrose used in RCM. In addition, pH indicators, such as phenol red, bromocresol green, and bromocresol purple were added to the BEM medium in an attempt to increase the efficiency of recognition of bifidobacterial colonies. Again, it was assumed that other enteric organisms would be present and able to ferment the lactose, but a yellow zone combined with the colonial morphology would help with bifidobacterial recognition.

A comparison of B50, a published selective medium, with various bifidobacterial enumeration media can be seen in Figures 2.3.7, 2.3.8, and 2.3.9. Although none of the BEM formulations appeared to enumerate bifidobacteria more effectively than B50, the addition of a pH indicator, such as phenol red, in the bottom agar layer did help in the recognition of bifidobacterial colonies during the counting process. This can be clearly seen in Figure 2.3.9. Both BEM6B and BEM6C enumerated a significantly higher number of *total* bacteria. BEM6C also enumerated a significantly higher number of presumptive bifidobacteria. However, BEM6C also enumerated a significantly higher number of background colonies compared to B50. BEM6C allowed more differentiation of bifidobacterial colonies, but was not as selective as B50.

Of all the BEM formulations tested, BEM6C appeared to be the most effective. In order to decrease the substantial background population, some or all of the selective agents used in the agar overlay should be incorporated in the bottom agar

layer. The most effective addition would probably be iodoacetate, as this compound blocks glycolysis, in response to which bifidobacteria can utilize the fructose-6-phosphate shunt (35). However, as most of the bifidobacteria present are injured, a lower concentration of iodoacetate should be used.

A quality control step was employed to check if the colonies that were considered presumptive bifidobacteria actually could be confirmed as *Bifidobacterium* spp. Colonies were randomly picked from the selective plates and cultured in RCM. The procedure shown in Figure 2.2.2 was used in the identification of the isolates as members of the genus *Bifidobacterium*. Ten of these isolates gathered from the constructed wetlands and primary treated sewage were examined for their carbohydrate fermentation patterns. All ten isolates keyed out to be bifidobacterial species of a possible human origin. The most interesting note here is that none of the isolates keyed out to *B. bifidum*, one of the most ubiquitous species of bifidobacteria.

Conclusions and recommendations. Bifidobacteria generally show promise in their use as fecal pollution indicators. The results from Chapter 1 show that bifidobacteria can be detected in aquatic environments for a short time following inoculation and are incapable of aftergrowth in these environments even when anoxic conditions prevail. The results from this chapter show that bifidobacteria are readily enumerated from constructed wetlands receiving primary treated sewage. The bifidobacterial populations detected in the wetlands therefore indicate recent and ongoing input in order to maintain the population levels detected – especially when the wetlands contained aerobic conditions. This would help explain the lower variation in

bifidobacterial populations over time compared to the fecal coliforms and enterococci. The fact that the fecal coliforms and enterococci were also being 'replenished' each time the influent was turned on and yet they exhibited a wide range of variability in their populations over time is puzzling. If levels of fecal coliforms or enterococci (both accepted fecal pollution indicators) ever became undetectable in the effluent from these wetlands, a false sense of water quality could be imagined.

Although bifidobacteria were readily enumerated from the wetlands with the published selective media, those media exhibited insufficient sensitivity and selectivity to efficiently enumerate bifidobacteria. This point has been a major hurdle in the use of bifidobacteria as water quality indicators. Results from Chapter 1 show a relatively quick temperature-dependent injury rate for bifidobacteria in aquatic environments. These injured cells became nonviable within a short amount of time even when trying to enumerate them onto a nonselective, nutrient-rich agar. Attempts to improve upon the published selective media were only successful in improving the ability to differentiate presumptive bifidobacterial colonies from the ever-present background populations. Improving the ability to enumerate injured bifidobacteria from the wetland samples as well as increasing the selectivity of the media were mixed to unsuccessful. The actual population of bifidobacteria in the wetlands therefore may have been an order of magnitude higher or more.

Another major disadvantage to using bifidobacteria as fecal pollution indicators is the amount of time involved to get a confirmed result. In the public health arena, time is of the essence when protecting the well being of the general public. Routine monitoring of drinking water systems, and ground water and surface

water is done to prevent the appearance or spread of illnesses that historically have claimed many lives. The 2 to 3 week timeframe for even *one* sample to be confirmed positive for bifidobacteria is too long to adequately protect the public health. Yet, we are constrained by the long generation time that bifidobacteria exhibit – 4 to 5 hours on average. In order for this genus to be effectively used as a fecal pollution indicator, a non-cultural method must be found.

It may help to develop a presence-absence test like that for coliforms to avoid the problem of absolute quantification. Since bifidobacteria are exclusively of fecal origin, if these organisms are present, it really does not matter how many there are, only that they are there. If a presence-absence test is adequate, another option could be to use 16s DNA and the polymerase chain reaction coupled with bifidobacterial-specific primers to monitor water quality. This method is well documented in the literature for other organisms and other environments and can be concluded within a day of taking the water sample. There are species-specific primers for bifidobacteria published in the literature (8,25,26,32,59).

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In August 1988, he enrolled at Frostburg State University in Frostburg, Maryland, where he received a Bachelor of Science degree in Biology in 1992. During this time he also worked as a student technician in the Environmental Health Division of the Allegany County Health Department. From 1992 to 1995 he was enrolled in the graduate program at West Virginia University in the Department of Plant Pathology and Environmental Microbiology pursuing a Master of Science degree.

In August 1995, he began graduate studies at Michigan State University in the Department of Crop and Soil Sciences pursuing a Doctor of Philosophy degree in soil microbiology. He officially became a candidate for the degree of Doctor of Philosophy in January, 1999.