DEICING/ANTI-ICING FLUID DEGRADATION AND ETHYLENE GLYCOL UPTAKE BY A PSYCHROTROPHIC *PSEUDOMONAS FLUORESCENS*

by

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Abstract

The psychrotrophic *Pseudomonas fluorescens* used in this study demonstrated the ability to grow upon and utilize ethylene glycol and propylene glycol at 25°C and 5°C. Growth at both temperatures was marginally quicker with propylene glycol; but acclimation was faster when ethylene glycol was the substrate. Propylene glycol was more persistent than ethylene glycol at both 25°C and 5°C. When both substrates were present in the same reaction system, both were utilized concurrently, however, the relative recalcitrance of propylene glycol was still observed.

The ability of the microorganism to biodegrade both clean and spent Union Carbide UCAR XL-54 (a deicer) and UCAR Ultra (an anti-icer) was shown. Growth occurred at 25°C at concentrations ranging from 10,000 mg/L to 100,000 mg/L (ethylene glycol), illustrating the ability of the microorganism to withstand and grow upon high concentrations of these formulations. At the lower concentrations, the generation times were quite similar to those obtained on pure ethylene glycol, suggesting that the additives present in these formulations were not particularly inhibitory to the microorganism. Utilization of the ethylene glycol in the clean UCAR deicing/anti-icing fluids (initial glycol content of 10,000 mg/L) was slightly enhanced compared to pure ethylene glycol at both 25°C and 5°C.

Spent deicing fluids collected from Pearson International Airport in Toronto, Ontario were also shown to be readily biodegraded by the psychrotroph. The spent fluids were diluted to approximately 10,000 mg/L after which growth and utilization were observed at both 25°C and 5°C. The spent fluids contained a natural population of microorganisms which were capable of ethylene glycol degradation at 25°C; however, at 5°C, they were inactive. These findings indicate the importance of using psychrotrophs in low temperature applications. It was also determined that to promote the utilization of the spent fluids by either the natural population or the *P. fluorescens*, inorganic supplements were required.

Physiological studies investigating enzyme induction and substrate uptake indicated that ethylene glycol utilization and uptake are susceptible to external variables. Growth of

the microorganism on propylene glycol did not induce the oxidase necessary for the oxidation of ethylene glycol into glycolate. This suggests that the oxidase activity involved in the initial oxidation of propylene glycol and ethylene glycol originates in two separate enzymes. Despite this, propylene glycol-grown cells were capable of inducing ethylene glycol uptake, however, the relative uptake was less then that observed for ethylene glycol grown cells.

The ethylene glycol transport mechanism was found to be induced by certain monohydric and dihydric alcohols. The ability of these alcohols to induce transport was inversely related to the chain length of the alcohols tested. It was shown that non-growth supporting monohydric and dihydric alcohols were capable of competing with ethylene glycol for the transport mechanism. Similar findings were found with diethylene glycol, also a non-growth supporting substrate. It thus appears that this transport mechanism is not exclusive for ethylene glycol.

The use of 2,4-dinitrophenol and N,N'-dicyclohexylcarbodiimide indicated that the uptake mechanism was energy dependent. This suggests that ethylene glycol transport may involve a secondary active transport process such as symport.

Environmental factors such as pH and temperature influenced ethylene glycol uptake and it was determined that maximal uptake occurred at pH 7 and 25°C. Although the uptake decreased with a drop in temperature, it was significant that ethylene glycol uptake was still occurring at 0°C.

Culture age and starvation were found to affect uptake and it was shown that cells in the early exponential phase of growth as well as those which were not subjected to nutrient limitation were capable of greater rates of ethylene glycol uptake.

In summary, the psychrotrophic *P. fluorescens* demonstrated the ability to utilize clean and spent UCAR deicing/anti-icing fluids and the degradation rate may be influenced by external factors through their effect upon the uptake of ethylene glycol into the cell.

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"I mish d'it" (I'm finished)

Matteo De Francesca (age 1)

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1. Introduction

1.A. Ethylene glycol, propylene glycol and aircraft deicing/anti-icing fluids

1.A.1. General overview

Ethylene glycol and propylene glycol belong to a group of chemicals known as dihydric alcohols, or more simply, diols. Diols are aliphatic compounds that contain two hydroxyl moieties in the molecule; the structure of these compounds is described by the following formula: $C_nH_{2n}(OH)_2$ (Monick, 1968).

A wide variety of commercially important glycol derivatives (*i.e.* polyethylene and polypropylene glycols) are based on ethylene glycol and propylene glycol. As a result, these two glycols represent the most industrially important members of the diol family and are thus produced in high quantity. In 1996, the estimated Canadian annual production of ethylene glycol was 825,000 t. In 1992, the annual propylene glycol production was approximately 16,000 t. On a global scale, ethylene glycol production in 1996 was approximately 10.2 million tons with North America, Western Europe and Japan producing approximately half this amount. These three regions also represent the largest consumers of ethylene glycol with approximately 4.1 million tons being used for polyethylene terephthalate (PET) production and antifreeze formulations (SRI Consulting, 1996).

Glycols are used in a wide variety of industries with the greatest consumers being those manufacturing PET and antifreeze mixtures. Secondary uses include the processing of oil and gas and the manufacture of cellulose film, explosives and solvents (Jones and Watson, 1976; Dwyer and Tiedje, 1983; Canadian Council of Resource and Environment Ministers, 1995). With such a wide array of uses, glycols inevitably affect the environment. It was reported by Miller (1979; *In* Canadian Council of Resource and Environment Ministers, 1995) that the main sources for environmental contamination are glycol production, transportation, use and disposal, which basically covers all facets of glycol handling. In Canada, the most evident source of glycol release into the

environment is the air transport industry, which together with automotive coolant systems accounts for about 60% of the ethylene glycol used. (Canadian Council of Resource and Environment Ministers, 1995).

1.A.2. Glycol use in the air transport industry

The air transport industry establishes regulations which assure the safe operation of aircraft during cold weather. According to Canadian Air Regulation 540.2 of the federal *Aeronautics Act*, "No aircraft will attempt a takeoff with any ice or snow adhering to its flight control surfaces" (Air Transport Association of Canada, 1994). In the United States, Federal Aviation Regulations prohibit the operation of an aircraft when frost, snow or ice is adhering to "any propeller, windshield, or powerplant installation or to an airspeed, altimeter, rate of climb, or flight attitude instrument system." In addition, the regulations prohibit operation when ice or snow is "adhering to the wings or stabilizing or control surfaces," or when frost is "adhering to the wings or stabilizing or control surfaces, unless that frost has been polished to make it smooth" (Duncan, 1995). The presence of snow, ice or frost on an airplane can disturb the airflow around the airframe structure. This ultimately results in reduced lift, increased drag, increased stall speed and abnormal pitch characteristics. What makes this exceedingly dangerous is that accumulation only as thick and rough as medium-grit sandpaper is enough to alter the aerodynamic behaviour of an aircraft. To ensure that icing conditions are not altering the predictable flow of air around the airframe, aircraft deicing/anti-icing fluids are utilized (Duncan, 1995).

Ethylene glycol, propylene glycol and to a lesser extent, diethylene glycol are primary components of numerous aircraft deicing/anti-icing fluids. During periods of adverse winter weather, the volume of these fluids used can be exceptionally high. Based on the 1989-1990 winter season, the total usage of deicing/anti-icing fluids in the United States amounted to 43.5 million litres. In Michigan, the total usage was in the range of 3.1 to 4.2 million litres with approximately 3 million litres being used at Detroit Metropolitan Airport. This works out to a September-through-April daily average of 12,140 L/day

(Sills and Blakeslee, 1992). A single large aircraft can require thousands of litres of deicing/anti-icing fluids to meet federal safety regulations. Large commercial aircraft such as a DC-9 or a Boeing 727 may require anywhere from 800 to 15,000 litres of deicing/anti-icing fluid in a single application (Sills and Blakeslee, 1992). The US Environmental Protection Agency (EPA) has reported that the annual ethylene glycol use at 35 American airports surveyed ranged from 5685 litres to greater than 17 million litres per airport (Natural Resources Defense Council, Inc., 1997). In Canada, the release of ethylene glycol by airports is required to be reported according to regulations set by the Canadian National Pollutant Release Inventory (NPRI). According to NPRI, 6 of the top 10 facilities reporting the release of ethylene glycol are airports or aviation service companies. These 6 facilities alone were responsible for the release of more than 1 million litres of ethylene glycol. Furthermore, among the chemicals listed in the NPRI, ethylene glycol ranks 9th with respect to volume released (Natural Resources Defense Council, Inc., 1997). In the American equivalent to the NPRI, the Toxic Release Inventory (TRI), ethylene glycol release reporting by the air transport industry is not required. However, in the event that airports were required to provide chemical release information, the volume of ethylene glycol used would result in the air transport industry being ranked 9th out of 20 industries reporting to the TRI (Natural Resources Defense Council, Inc., 1997).

Ethylene glycol and other glycols used in deicing/anti-icing fluids are unique in that their use dictates that they be released into potentially non-confined areas. Consequently, environmental contamination resulting from runoff has become an important issue. For example, in surface waters located near airports, ethylene glycol levels as high as 13,200 mg/L have been reported (Canadian Council of Resource and Environment Ministers, 1995). Recent Transport Canada reports for Halifax, Mirabel and Pearson International airports have ethylene glycol concentrations in surrounding surface waters in the range of <10 to 643 mg/L (Canadian Council of Resource and Environment Ministers, 1995). The environmental fate of ethylene glycol must therefore be considered.

1.A.3. Ethylene glycol and propylene glycol degradation

When glycols reach the environment, they are primarily degraded by either aerobic or anaerobic processes (See Appendix for illustration). The former appears to contribute more significantly to glycol utilization. The aerobic biodegradation of glycols has been studied by a number of groups and the results suggest that ethylene glycol and propylene glycol are relatively nonpersistent (Boatman *et al.*, 1986; Child and Willetts, 1978; Colucci and Inniss, 1996; Evans and David, 1974; Fincher and Payne, 1962; Gonzalez *et al.*, 1963; Haines and Alexander, 1975; Jones and Watson, 1976; Kaplan *et al.*, 1982; McVicker *et al.*, 1998; Pearce and Heydeman, 1980; Thelu *et al.*, 1980; Wiegant and DeBont, 1980; Willetts, 1981). It has also been found that the utilization of ethylene glycol is temperature and acclimation dependent (Colucci and Inniss, 1996; Cox, 1978; Dwyer and Tiedje, 1983; Evans and David, 1974; Williams, J., Union Carbide Ltd., unpublished data, *In* Canadian Council of Resource and Environment Ministers, 1995).

Anaerobic biodegradation of glycols is considerably slower than aerobic processes (Cox, 1978; Dwyer and Tiedje, 1983; Kaplan *et al.*, 1982; Pearce and Heydeman, 1980; Schink and Steib, 1983). Consequently, when glycols are present in anoxic environments, they may persist longer than in surface waters. In fact, it has been suggested that biodegradation rates of ethylene glycol in subsurface environments would be half of those recorded in surface waters (Syracuse Research Corporation, 1989, *In* Canadian Council of Resource and Environment Ministers, 1995).

To date, the only water quality guidelines which exist for ethylene glycol and propylene glycol are those for receiving waters and they are set at 3 and 74 mg/L, respectively (Canadian Council of Resource and Environment Ministers, 1994). However, a greater concern for the environment than the straight toxicity of the glycols is their high oxygen demand. It is possible that the biodegradation of glycols can be sufficiently quick that environments which are normally aerobic can quickly shift to anoxic conditions. This presents a hazard to aquatic life, especially in ice-covered water bodies where oxygen

depletion could last until the ice cover breaks. Therefore, in addition to the toxicity of glycols, it is important to assess the effects of glycol degradation upon the dissolved oxygen levels in receiving waters (Canadian Council of Resource and Environment Ministers, 1995).

1.A.4. Aircraft deicing/anti-icing fluid degradation

Most of the existing studies regarding glycol biodegradation are based on various types of defined media supplemented with pure glycols. While this provides valuable information regarding the ability of microorganisms to utilize these glycols, it does not address the issue of formulated glycol-based products such as aircraft deicing/anti-icing fluids. It has been found that these fluids are more toxic than pure glycols and this may be due to additives present in these products (Canadian Council of Resource and Environment Ministers, 1995; Pillard, 1995). Therefore, it is necessary to investigate the ability of microorganisms to withstand the toxicity of deicing/anti-icing fluids and subsequently biodegrade them.

There is limited information regarding the biotreatment of glycol-based formulations such as deicing/anti-icing fluids. In a study conducted by Sabeh and Narasiah (1992), a mixed culture originating from sludge taken from an aerated pond demonstrated the ability to utilize deicing fluids at 4°C, 10°C and 22°C. Similar findings were reported by Klecka *et al.* (1992) where several aircraft deicing fluids (Dow 146AR, 1000PG, Flightgard 2000 (FG2000), Association of European Airlines type I (AEA-1) and Texaco WD-20) were shown to be biodegradable in soil from –2°C to 25°C. Strong-Gunderson *et al.* (1995) reported on the ability of a microbial consortium to biodegrade high concentrations of glycol-based waste including deicing fluids but unfortunately, experiments were conducted at 25°C. Nitschke *et al.* (1996) also reported the biological treatment of waste water containing glycols from deicing agents, however, the deicing agents used were not ethylene glycol-based formulations.

In Canada, the primary fluids used are ethylene glycol formulations manufactured by Union Carbide. These fluids are divided into two types (Type I and Type II). Type I fluids are approximately 50% ethylene glycol and 2% additives in water. This fluid is heated to 85°C and used to remove snow and ice from the flight control surfaces. As a result, Type I fluids are generally known as deicing fluids. These fluids may also be diluted for less severe icing conditions; however, this limits their effectiveness (Sills and Blakeslee, 1992). Type II fluids are based on 40-50% ethylene glycol with up to 30% additives. A component of the additives is a polymer-based thickening agent which helps the fluid to remain on the aircraft until a minimum shear force is attained during takeoff. This fluid is sprayed unheated after application of the Type I fluid (the deicer) and serves to prevent the accumulation of ice or snow upon the deiced aircraft. As a result, these fluids are referred to as anti-icing fluids (Sills and Blakeslee, 1992). At Pearson International Airport Trillium Terminal 3 (Toronto, Ontario, Canada), the Type I and Type II fluids used are UCAR XL54 and UCAR Ultra, respectively.

1.B. Cold-adapted microorganisms

1.B.1. Psychrotrophs and psychrophiles

Temperature is one of many factors upon which microorganisms are commonly classified. By determining the range of permissible growth temperatures, microorganisms are generally divided into three broad categories: psychrophiles, mesophiles and thermophiles. In general, the growth temperatures for psychrophiles, mesophiles and thermophiles are -5 to 20°C, 15-40°C and 45-100°C, respectively (Herbert, 1992). In addition to these three categories is the class of psychrotrophs that roughly fits between the psychrophiles and mesophiles. Psychrotrophs and psychrophiles are very similar in that both classes are well adapted to cold climates. The primary difference between the two is that while both classes demonstrate growth at 0°C or less, psychrophiles have a maximum growth temperature of 20°C or less while psychrotrophs maintain a maximum growth temperature which is greater than 20°C (Morita, 1975).

The Earth is essentially a cold planet. Although man inhabits the more moderate regions, more than 80% of the Earth's biosphere is permanently cold (Russell, 1990). This is due to the fact that more than 70% of the Earth is covered by seawater of which more than 90% is below 5°C (Ludlow and Clark, 1991). Within this environment, psychrotrophs and psychrophiles can be found in solution, attached to debris, within other organisms and in sediment. Additional reservoirs for these microorganisms are Arctic and Antarctic soil, snow, deserts, and glaciers (Ludlow and Clark, 1991; Feller *et al.*, 1996). More recently, however, psychrotrophs have been isolated from atypical environments such as tropical soils. It was reported by Astwood and Wais (1998) that a number of isolates were cultured from the soils of the southeastern coastal zone of Jamaica and these isolates were capable of growth at 2°C. One strain in particular demonstrated the ability to grow over the very wide temperature range of 2°C to 44°C.

1.B.2. Physiological adaptations for growth at low temperature

Psychrotrophs and psychrophiles are capable of growth at lower temperatures due to numerous adaptations. The adaptations allow microorganisms to virtually maintain all of the same biochemical processes noted during growth at moderate temperatures (Margesin and Schinner, 1994).

Enzymatic processes are largely dependent upon temperature; the rate of enzymatic reactions can be expressed by the Arrhenius equation (Gounot, 1991).

$$k = Ae^{-Ea/RT}$$

k	=	reaction rate constant
Ea	=	energy of activation
A	=	a constant related to steric factors and collision frequency
R	=	universal gas constant
T	=	absolute temperature

Based on the above equation, a temperature decrease translates into a decrease in the reaction rate constant (k). Within a living cell, this is often noted by a decrease in growth rate. An interesting observation is that when psychrotrophs and psychrophiles are subjected to a temperature decrease, the rate of decrease in the cells' overall activity is

slower than mesophilic cells subjected to the same treatment (Margesin and Schinner, 1994). In other words, mesophiles exhibit changes in activity that correspond to the above thermodynamic relationship, whereas psychrotrophs and psychrophiles demonstrate a deviation from the predicted results. Researchers have found that these microorganisms exhibit certain enzyme adaptations which compensate for the effects of temperature upon enzyme reactions. These adaptations are usually classified as either qualitative or quantitative. Qualitative changes involve the induction of enzymes that are better suited to the colder temperatures. Quantitative changes generally refer to alterations in the enzyme concentration. By altering the types and/or concentrations of the enzymes present, the microorganism is able to maintain vital cellular processes at the lower temperatures (Russell, 1990).

Enzymes from psychrotrophs and psychrophiles are less sensitive to cold temperatures and exhibit higher catalytic efficiencies compared to mesophilic equivalents. This is achieved through a more flexible structure which demands less activation energy for a reaction to occur (Feller *et al.*, 1996). This flexibility alters enzyme conformations and leads to potential variations in substrate affinities as well as in interactions of the enzymes with allosteric effectors or inhibitors. The structural modifications found in cold-adapted enzymes generally result in an optimal enzymatic activity that is shifted to lower temperatures; this appears to be accompanied by an increase in heat sensitivity (Margesin and Schinner, 1994; Feller *et al.*, 1996).

To compensate for decreased enzymatic activity, psychrotrophs and psychrophiles may increase the synthesis of certain enzymes. The effect seems to be more dependent upon the growth temperature rather than the defined optimal growth temperature particular to the microorganism in question (Feller *et al.*, 1990; Gügi *et al.*, 1991).

When microorganisms experience sudden downshifts in temperature, they produce cold shock proteins (csps). The roles of csps in mesophiles, psychrotrophs and psychrophiles are under investigation but it appears that csp induction is linked to

transcriptional and translational processes as well as mRNA degradation (Jones *et al.*, 1987). Broeze *et al.* (1978) noted that at temperatures below the minimum required for growth, there was a blockage in the initiation of translation which led to polysomal runoff and the accumulation of ribosomal subunits. Tai *et al.* (1973) has suggested that blockage of the initiation of translation may be the cause of growth termination during a decrease in growth temperature. What is interesting is that with respect to the initiation of protein synthesis, psychrotrophs and psychrophiles are less affected by decreases in temperature compared to mesophiles (Broeze *et al.*, 1978). This observation suggests that the proteins involved in protein synthesis are specialized to be operational at the lower temperatures, a factor resulting from the qualitative or quantitative alterations mentioned previously. Additionally, the ribosomes of some psychrotrophs differ from those of mesophiles and this alteration may influence the translation process in some manner (Bertoli and Inniss, 1978). To accommodate the demand and synthesis of specialized proteins, psychrotrophs and psychrophiles exhibit a rapid protein turnover rate to keep the amino acid supply as healthy as possible (Potier *et al.*, 1985).

The favorable effect of aeration upon psychrotrophs and psychrophiles is another feature that distinguishes these microorganisms from mesophiles. Lower temperatures increase the solubility of oxygen; as a result, more oxygen is available to the microorganism. Since psychrotrophs and psychrophiles are adjusted to operate under conditions of increased oxygen, increases in aeration have a greater effect upon these microorganisms compared to mesophiles (Margesin and Schinner, 1994).

Changes in membrane structure are also demonstrated by psychrotrophs and psychrophiles (Gounot, 1991) as lipid-based membranes are susceptible to changes in temperature. When the temperature decreases below the gel-liquid-crystalline transition temperature, membranes shift from a fluid state towards a more crystalline state. This has a negative impact on membrane-related activities such as solute transport (Sikkema, 1995). Psychrotrophs and psychrophiles are able to maintain vital membrane processes

due to several adaptive features. Firstly, membranes of psychrotrophs and psychrophiles contain a higher lipid content compared to mesophiles. Russell and Fukunaga (1990) have suggested that, at lower temperatures, the increased lipid content may result in larger cells with greater surface area and consequently increased nutrient uptake. Secondly, the level of unsaturated fatty acids is increased when growth occurs at lower temperatures (Neidleman, 1990). The presence of unsaturated fatty acids leads to a decrease in the lipid melting point, thus aiding to maintain membrane fluidity at the lower temperatures. Without membrane fluidity, membrane transport would be negatively affected and the microorganism would be starved of nutrients and excretion products would not be properly removed from the internal environment of the cell.

1.B.3. Use of psychrotrophs and psychrophiles in biodegradation

Since the majority of the Earth's biosphere is permanently cold, it follows that many of the biotransformations which occur in nature happen at lower temperatures. Unfortunately, within industrial applications, the issues of time and efficiency dictate that biotransformations occurring at lower temperatures are too slow. Since thermodynamics suggest that with increased temperature there in an increase in enzymatic rate, enzymes and/or microorganisms which are suitable for processes occurring at higher temperatures have been the focus of most biotechnological research.

More recently there has been a drive towards energy conservation and cost-savings. This shift has opened up new interest in the capabilities of psychrotrophic and psychrophilic microorganisms. Psychrotrophs have long since been identified as industrially important organisms within the food and beverage industry, however, their use in bioremediation has been somewhat limited. The current literature indicates that the trend is changing; psychrotrophs are increasingly being considered in various biodegradative processes.

A common misconception regarding the use of psychrotrophs in bioremediation is that their overall metabolic activity is governed solely by thermodynamics. It has been

assumed that biotransformations occurring at lower temperatures can be predicted by applying the Arrhenius equation to similar known processes occurring at elevated temperatures. The general rule followed is that for every 10°C decrease in temperature, there is a 50% decrease is metabolic activity (Bradley and Chapelle, 1995). In groundwater systems, it is expected that the greatest overall microbial activity occurs in the range of 20°C to 40°C, however, groundwater systems are rarely within this temperature range. Therefore, it is assumed that contaminated groundwater systems, which may have an average temperature below 10°C, will show little if any metabolic activity. The misconception is particularly a problem with Arctic sites where the rates of biodegradation by the indigenous population are predicted to be too slow for effective contaminant removal (Whyte, *et al.*, 1998). This assumption is incorrect since microbial adaptation to reduced temperatures is more complicated than the simple application of thermodynamics to known processes.

To properly assess the effectiveness of a low temperature biodegradative process, it is preferable to design experiments which simulate the low temperature environment. Although the number of studies conducted with psychrotrophs fails to compare to those with mesophiles, low temperature biodegradation has been reported for a variety of compounds including polychlorinated biphenyls (Harkness *et al.*, 1993; Mohn *et al.*, 1997; Master and Mohn, 1998), crude/diesel oils (Swannell *et al.*, 1996; Lal and Khanna, 1996; Margesin and Schinner, 1997a; Margesin and Schinner, 1997b), phenols (Kotturi *et al.*, 1991; Jarvinsen *et al.*, 1994), naphthalene (Whyte *et al.*, 1996; Whyte *et al.*, 1997) and various hydrocarbons including benzene, toluene, dodecane, hexadecane and octane (Whyte *et al.*, 1996; Whyte *et al.*, 1997; Whyte *et al.*, 1998).

Polychlorinated biphenyls (PCBs) have not been produced in the United States or Canada since the mid-seventies, yet, they remain a concern in North America due to numerous sites polluted with these compounds. Many of these sites are in the Arctic or sub-Arctic regions and due to their remote locations, cleanup using conventional

physical/chemical methods is very expensive (Master and Mohn, 1998). The PCBs in polluted low-temperature environments set up selective pressure towards the presence of cold-adapted PCB-degrading microorganisms (Mohn *et al.*, 1997). In a treatment scheme designed to operate at ambient temperatures, the utilization of psychrotrophs or psychrophiles producing cold-adapted enzymes presents an advantage over mesophiles and their enzymes. In studies conducted by Mohn *et al.* (1997) and Master and Mohn (1998), the PCB degrading potential of psychrotolerant microorganisms was demonstrated. It was found that Arctic soil microorganisms isolated from a PCB-contaminated site were capable of degrading all mono-, most di- and some trichlorobiphenyl congeners at 7°C. Two isolates were especially active at 7°C with removals of Aroclor 1221 in the range of 54% to 60%. In general, the Arctic soil microorganisms showed higher degradative capacity at 30°C compared to 7°C, however, the differences in activity were considerably less than what would be defined by thermodynamics. This supports the idea that although temperature will influence reaction rates, it does not define the ultimate microbial activity observed. It was also found that PCB degraders could be isolated from the PCB-contaminated soils but none were found in pristine locations. This finding supports the idea that polluted environments will select for microorganisms capable of adapting to their surroundings. In this particular research, the microorganisms isolated were most closely related to pseudomonads (Master and Mohn, 1998). The use of psychrotrophs and psychrophiles in more temperate regions would also be advantageous. In a PCB biodegradation study conducted in the Hudson River (New York), it was determined that the river water temperature ranged from 28°C (August) to 10°C (October), at which time the experiment was terminated. Biodegradation of PCBs was noted during the 73 day study; however, additional PCB removal may have been possible throughout the winter months if psychrotrophs and/or psychrophiles were also involved. Unfortunately, this was not the focus behind the study (Harkness, *et al.*, 1993).

The low temperature biodegradation of crude and diesel oils probably represents the most widely studied area in the application of psychrotolerent microorganisms. The amount of petroleum used in the world exceeds $4x10^{12}$ litres a year and it is estimated that approximately 0.1%, or $4x10^{9}$ litres, is released directly into the environment (Prince, 1992). Fortunately, biodegradation of petroleum and hydrocarbon products is common and represents the primary mode of environmental decontamination (Leahy and Colwell, 1990). Much of this decontamination is occurring at lower temperatures. Despite this, many of the studies conducted have been performed in the range of 20°C to 30°C which is not representative of many of the environments where petroleum contamination is a problem. For example, alpine environments have an average annual temperature in the region of 7°C. Fortunately, the use of psychrotrophs at ambient temperatures is receiving increased attention (Margesin and Schinner, 1997a). Earlier studies indicated that bacteria isolated from Chesapeake Bay were capable of degrading a model petroleum at both 0°C and 5°C (Walker and Colwell, 1974). Similar low temperature degradation was reported by Westlake *et al.* (1974) and Atlas (1981). More recent studies by Margesin and Schinner (1997a, 1997b) indicate that diesel oil biodegradation is successful at 10°C in alpine subsoils. It was also noted that the rates of decontamination were similar to published rates measured at 25°C and 30°C.

Polluted environments have shown to be excellent reservoirs of bacteria with specific and desirable degradative mechanisms. Whyte *et al.* (1996) screened 135 psychrotrophic strains isolated from northern sites for their ability to biodegrade toluene, naphthalene, dodecane and hexadecane. The results indicated that numerous strains were able to degrade one or more of the above compounds at both 23°C and 5°C. PCR and Southern hybridization experiments indicated that some of the psychrotrophic strains contained genes that hybridized to probes for known genes (*i.e. todCl*, *xylE* and *ndoB*) in mesophiles with aromatic and polyaromatic degradative abilities. This suggests that some of the catabolic pathways characterized for mesophiles may be present and active in

psychrotrophs and psychrophiles. However, novel catabolic mechanisms may also be present; it was reported by Whyte *et al.* (1996) that two toluene degraders tested negative for the presence of *xylE* and *todCl*, genes normally associated with toluene degradation pathways. It was further reported by Whyte *et al.* (1997) that two psychrotrophic pseudomonads, isolated from petroleum-contaminated Arctic soils, tested positive for the ability to biodegrade both aliphatic (C_5 to C_{12} n-alkanes) and polyaromatic hydrocarbons (toluene and naphthalene) at 25°C and 5°C. There are very few reports that describe this combined catabolic ability and it appears that this report is the first to describe a strain containing both the *alk* and *nah* pathways located on two different plasmids within a naturally occurring bacterium. In 1998, Whyte *et al.* described a psychrotrophic *Rhodococcus* sp. which had the ability to utilize a variety of short and long chain alkanes in addition to various aliphatics (C_{10} to C_{21} alkanes, branched alkanes, a substituted cyclohexane) present in diesel fuel at 5°C. The alkane specificity of the microorganism paralleled that found in many other characterized mesophilic alkane-degraders.

Phenols and chlorophenols have also been documented as being biodegraded at low temperatures. Kotturi *et al.* (1991) found that *P. putida* Q5 was capable of phenol degradation at 10°C; as expected, the rates were slower than comparable studies conducted with mesophiles at 30°C. Jarvinsen *et al.* (1994) reported on the biodegradation of chlorophenols, a group of compounds found to be quite recalcitrant in nature. An aerobic fluidized-bed treatment scheme was used to decontaminate chlorophenol polluted groundwater. Removals of 99.9% were achieved at temperatures ranging from 4°C to 10°C. The treatment produced an effluent which contained only slightly greater concentrations of chorophenols than what would be permissible for drinking water.

In much of the biodegradation research presented, there is interest in the abilities of the natural population to perform the desired task of microbial decontamination. Numerous studies have investigated microorganisms isolated from contaminated sites. Master and Mohn (1998) determined that PCB degrading microorganisms were only found

in PCB contaminated soils and similar findings were reported by Whyte *et al.* (1996) with respect to hydrocarbon degraders. In the search for low temperature degraders, it becomes evident that northern contaminated environments would place the correct selective pressure upon the natural microbial population. However, the natural populations also have the advantage of being potentially adapted to various other factors which may be unique to a particular location. For example, microorganisms must also contend with pH, oxygen content, nutrient availability and soil characteristics (Margesin and Schinner, 1997b). In general, the indigenous population may be in a higher state of readiness compared to microorganisms prepared in a laboratory. The adaptations allowing for this may occur *via*: 1. induction/repression of specific enzymes, 2. genetic alterations and 3. selective enrichment of the natural population (Leahy and Colwell, 1990).

With the knowledge that contaminated sites may lead to the selection of the appropriate pollutant degraders, there is increasing debate regarding the practices of biostimulation versus bioaugmentation. In many contaminated environments, the availability of inorganic nutrients is often considered a major limitation. It has been noted that the addition of fertilizers often leads to enhanced microbial decontamination; thus the term biostimulation. In contrast, bioaugmentation refers to the practice of inoculating a contaminated site with a microbial preparation characterized in a lab under controlled conditions. For northern climates where the average temperature is below that appropriate for mesophiles, such a consortium would likely consist of psychrotrophs and/or psychrophiles. There are strong arguments for each method of treatment, however, it ultimately depends upon the specific contaminated site. Margesin and Schinner (1997a) reported on the successful biodegradation of diesel oil at 10°C using biostimulation via inorganic fertilizers. In recently contaminated sites where the natural population has not had time to acclimatize to the pollutant, the practice of bioaugmentation may serve to decrease the lag time before biodegradation begins to occur (Whyte *et al.*, 1998). Quite often, the addition of an inoculum only serves to increase the rate of biodegradation at the

initial stages of treatment, after which the indigenous population becomes dominant. As a result, bioaugmentation may be useful in environments where the contaminant has a toxic effect upon the indigenous population (Margesin and Schinner, 1997a). In most cases, however, bioaugmentation must be accompanied by fertilization due to the general lack of nutrients in many environments. Treatment via bioaugmentation must also take into account problems associated with microbial transport to the targeted areas, competition with the indigenous population, susceptibility to toxins or predators as well as preferential utilization of non-target substrates (Margesin and Schinner, 1997a).

In some cases, however, it may appear that biostimulation or bioaugmentation with naturally occurring microorganisms is unsuccessful. In such cases, it may be advantageous to genetically alter a microorganism with the desired catabolic traits. The majority of catabolic pathways characterized originate from mesophilic microorganisms (Whyte *et al*, 1998). Fortunately, it appears that laboratory-induced genetic exchange between mesophiles and psychrotrophs is possible. Kolenc *et al.* (1988) reported the successful transfer of the toluene degradation plasmid pWWO from a mesophilic *P. putida* to a psychrotrophic strain of *P. putida*. The genes of the toluene catabolic pathway were expressed in the psychrotroph; toluene biodegradation was noted at temperatures as low as 0°C. Thus, engineered microorganisms may serve to broaden the range of compounds susceptible to degradation at both moderate and reduced temperatures.

There are clearly numerous advantages to using psychrotrophs in bioremediation. The most commonly mentioned advantage is the cost-saving related to the absence of heating in the treatment processes. Stinson *et al.* (1991) reported that at pilot scale, heating could amount to 42% of the total cost when the temperature increase was less than 10°C. At full scale, this could increase to 60%. In the fluidized-bed treatment system investigated by Jarvinsen *et al.* (1994), heating from 7°C to 25°C could increase the cost of treatment by a factor of 2.5. Psychrotrophs and psychrophiles would eliminate the need for heating; however, this would generally be accompanied by an increase in time required

for complete decontamination. Another advantage is that the environments from which these organisms are isolated select for populations which are capable of dealing with relatively harsh conditions (*i.e.* low nutrient availability, low water activity). These microorganisms may prove to be workhorses among the overall bacterial population. The production of cold-adapted enzymes also presents advantages to using psychrotrophs and psychrophiles in northern climates. The ability of these microorganisms to grow and function at the lower temperatures together with the increased catalytic activity of their enzymes at these temperatures makes them excellent candidates for low temperature applications.

There are also a number of concerns regarding low temperature bioremediation. Although ambient temperature biodegradation eliminates the need for heating, the increased time required for complete decontamination may not be suitable for some applications. In addition, the complexity of a contamination problem should not be underestimated. Westlake *et al.* (1974) reported that no single species of bacteria will be able to handle a broad range of contaminants. If a treatment scheme requires the addition of an inoculum to initiate decontamination, a consortium of bacteria is likely required which as a whole exhibits a broad range of catabolic abilities. This clearly applies to all bioremediation applications, not just those designed to operate at lower temperatures.

1.C. Substrate uptake

For many substrates, the first step in biodegradation is transport across the bacterial envelope. To better understand how substrates enter the cell, the concept of solute transport will be addressed.

1.C.1. Transport processes

The transport of solutes across the bacterial cell envelope is dependent upon the selective nature of the cytoplasmic membrane and can occur via several different mechanisms. In some cases, the solute is able to simply diffuse through the lipid membrane or freely pass through a protein channel. In other mechanisms, the solute is

modified as it passes through the membrane preventing that molecule from migrating back out. Some processes require cellular energy to fuel transport and in many of these cases, the solute is accumulated against a gradient (Konings *et al.*, 1981).

Simple translocation through the membrane is called passive diffusion; this form of solute transport does not require the expenditure of cellular energy. Transport is the result of a concentration gradient between the internal and external environment. Although it is favourable from an energetic point of view, it is quite slow and is incapable of solute accumulation (Konings *et al.*, 1981).

To accumulate a solute against a gradient, or in some cases to simply transport a solute across a membrane, energy is required. Processes in which energy expenditure is necessary are collectively termed active transport processes. Many of the early studies of active transport were conducted by Peter Mitchell (Rosen and Kashket, 1978; Mitchell, 1967). Mitchell hypothesized that while proteins (enzymes) in solution are active in all directions, proteins embedded in membranes are vectorial. Since membrane proteins are unable to move about freely in all axes, the substrates corresponding to these proteins must approach from a particular direction with subsequent release in another. Therefore, Mitchell stated that membrane bound proteins exhibit vectorial metabolism. Such proteins are generally not providing a catalytic function but rather are serving as "carriers" across the membrane. Under the concept of vectorial metabolism, three types of transport were identified: Group translocation, Primary active transport and Secondary active transport (Rosen and Kashket, 1978).

Group translocation is not considered true active transport since it results in the modification of the solute, however, it is usually placed within the classification of active transport since the process is energy dependent. Group translocation transport is not exceedingly common; the only well documented system is the bacterial phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) (Kundig *et al.*, 1964). In the PTS, sugars are phosphorylated as they are transported into the cell. This

chemical modification renders the sugar membrane-impermeable thus preventing efflux. Although energy dependent, the PTS does not consume ATP or utilize the proton-motive force since all energy is derived from phosphoenolpyruvate (PEP). Hence, the process is considered to be energy conserving (Konings *et al.*, 1981; Neidhardt, *et al.*, 1990).

The second type of vectorial metabolism, primary active transport, is fundamental to the energetics of active transport in general. Primary active transport involves two main processes: Electron transfer and ATPase activity. The main idea behind primary active transport is the extrusion of protons from the cell to establish a proton gradient; this forms the basis of what Mitchell termed the chemiosmotic theory (Rosen and Kashket, 1978).

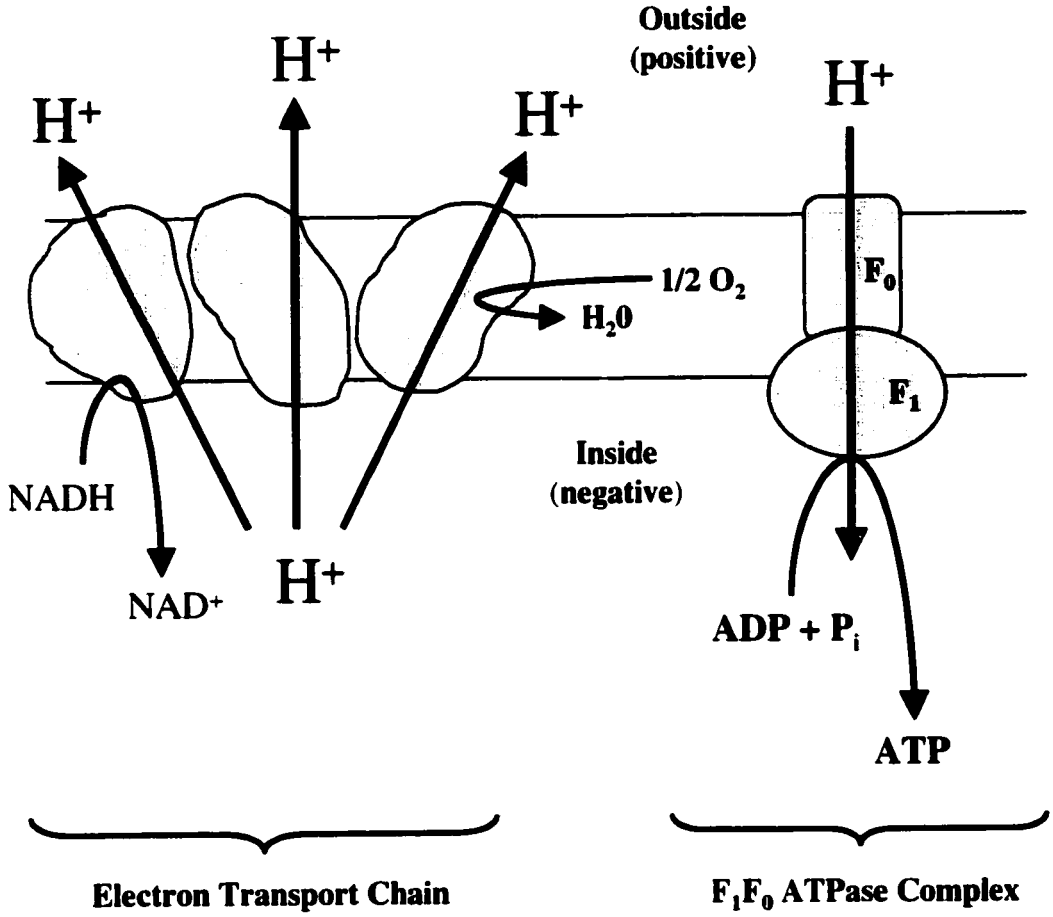
Electron transfer occurs via the electron transport chain. This process involves a series of electron transfers from the initial donor, e.g. NADH, to an eventual terminal acceptor, generally oxygen in aerobic environments. During the transfer process, protons are extruded from the cell. Since protons are unable to freely pass through the membrane, a proton-motive force is generated that consists of a membrane potential and a pH gradient. Mitchell's chemiosmotic theory then dictates that the proton-motive force is capable of driving ATP production through ATPase (Simoni and Postma, 1975). This process is represented in Fig. 1.

ATPase is a reversible enzyme complex which is capable of the following reaction:

$$ATP^{4-} + H_2O + nH^+_{in} \rightleftharpoons ADP^{3-} + P_i^- + nH^+_{out}$$

The catalytic direction of the ATPase complex is dependent upon the cellular environment. In general, under conditions of low proton-motive force and high (ATP)/(ADP), a proton-motive force can be generated by ATP-hydrolysis; whereas under conditions of low (ATP)/(ADP) and high proton-motive force, ATP-synthesis may occur (Konings *et al.*, 1981). The combined activities of the electron transport chain and the ATPase complex ultimately serve to supply the cell with the energy required to transport and accumulate solutes.

Figure 1: Chemiosmotic coupling of oxidation to phosphorylation. Protons are extruded from the cell via the electron transport chain and the resulting gradient is capable of driving ATP synthesis through the action of ATPase. (Adapted from Krulwich et al., 1998)


Outside
(positive)
H+
H+
H+
H+
1/2 O2
H20
F0
F1
NADH
NAD+
H+
Inside
(negative)
ADP + Pi
ATP
Electron Transport Chain
F1F0 ATPase Complex

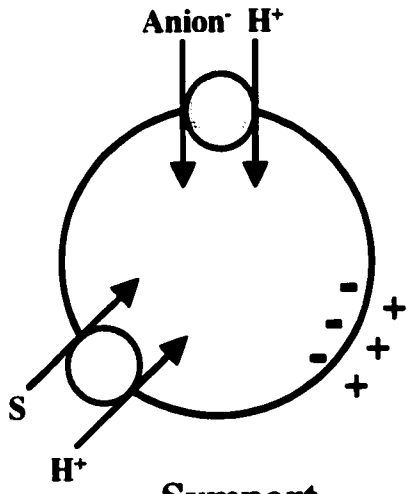
The transport of solutes into the cell with their subsequent accumulation is termed secondary active transport and this forms the third type of vectorial metabolism (Rosen and Kashket, 1978). The use of the term 'secondary' is used to indicate that the accumulation of a solute is at the expense of a previously established gradient (*i.e.* the proton-motive force). There are three types of secondary active transport: symport, uniport and antiport. These three transport mechanisms are illustrated in Fig. 2.

Symport transport refers to the cotransport of two substrates simultaneously in the same direction. In this process, the solute (*i.e.* an amino acid), is transported up its concentration gradient together with a proton, which is moving down its potential gradient. If the solute being transported is negatively charged (1:1 charge ratio with proton), the process is considered to be electroneutral with only the pH gradient contributing to transport. When the solute is neutral, the process is considered electrogenic in that both the pH gradient and the membrane potential, thus the total proton-motive force, contribute to transport (Rosen and Kashket, 1978).

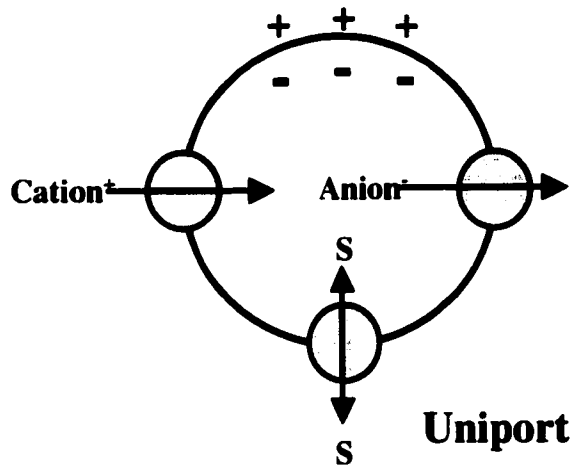
During uniport transport, the way by which a solute is translocated across a membrane is dependent upon its charge. For neutral substrates, the uniport systems acts as a facilitated diffusion system whereby transport is dependent solely upon the concentration gradient of the solute. Since a second gradient is not utilized, neutral solutes are unable to accumulate by this mechanism. When the solute is positively charged, the membrane potential dictates that the solute be translocated into the cell. Conversely, negatively charged molecules will be translocated out of the cell, again due to the membrane potential (Rosen and Kashket, 1978).

Antiport transport is similar to symport processes except that the two substrates being translocated are moving in opposite directions. As a result, antiport processes are responsible for the translocation of positively charged and neutral substrates. When the solute being translocated is positively charged (1:1 charge ratio with proton), the overall process is considered electroneutral. Under this circumstance, transport is driven by the pH

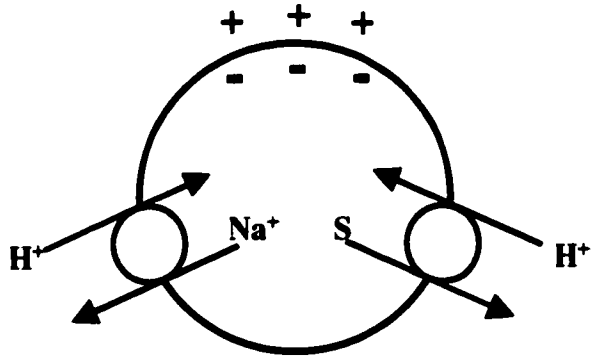
Figure 2: Secondary active transport: symport, uniport, antiport. During symport, anions and uncharged molecules (S) are transported in the same direction with protons, generally into the cell. During uniport, uncharged molecules (S) are passively transported, however, accumulation does not occur. Based on a negative internal environment, anions move out of the cell and cations are transported into the cell. Antiport involves the transport of cations and uncharged molecules (S), however, the solute and protons move in opposite directions. (Adapted from Rosen and Kashket, 1978)



Symport



Uniport



Antiport

gradient and the net movement of solute is out of the cell. For neutral solutes, the process is electrogenic and thus involves the entire proton-motive force (Rosen and Kashket, 1978).

In the above three secondary transport processes, the driving force behind accumulation of a solute is derived from primary active transport processes. If the proton-motive force were interrupted, for example through the use of uncouplers, these secondary active transport processes would cease to be active (Konings *et al.*, 1981). Primary active transport is responsible for the maintenance of the proton-motive force such that the energy required for secondary transport processes is available.

1.C.2. Carriers for secondary active transport

Solute transport relies on the activity of protein carriers to mediate the transport of molecules across the membrane. These proteins generally exhibit specificity for a restricted range of substrates. A large number of transport proteins have been identified and, using bacterial genome analysis, they have been organized into 67 families (Paulsen *et al.*, 1998). These families have been further organized into the following categories: channel proteins, secondary active transporters, primary active transporters, group translocators, and unclassified (Paulsen *et al.*, 1998). The secondary active transporter category consists primarily of symporters, uniporters and antiporters. In addition, this category contains the major facilitator superfamily, a family containing 17 subfamilies that mediate the transport of a wide variety of compounds including sugars, drugs, organophosphates, oligosaccharides, nitrate/nitrites, monocarboxylates, aromatic acids and various metabolites. Within each subfamily are numerous members which further indicate the vastness of protein transporters. For example, within the sugar porter subfamily, 133 members have been identified (Pao *et al.*, 1998). It is evident that the range of transport proteins in cells is extensive and there is increasing research towards understanding and classifying both known and newly identified members.

Secondary active transporters represent the largest category of transport proteins. Of the 67 families identified, 45 belong to this category (Paulsen *et al.*, 1998). The importance

of transport proteins to the proper maintenance and survival of bacteria cannot be underestimated. In *Escherichia coli*, 10.8% of the bacterial genome programs for transport proteins and of this, 24% and 28% is specific for nitrogenous and carbon compounds, respectively (Paulsen *et al.*, 1998).

Structure analysis of secondary active transporters indicate a relative simplicity in comparison to primary active transporters (*i.e.* ATPase). The average molecular mass is in the range of 40 to 55 kDa. The primary sequence consists of a hydrophobic polypeptide (approximately 20 amino acids long) that crosses the cytoplasmic membrane 12 times to form 12 α-helixes connected by hydrophilic loops that are longer on the cytoplasmic side of the membrane compared to the outside. The cytoplasmic hydrophilic loops contain a surplus of amino- and carboxy-termini that serve to determine the orientation of the hydrophobic transmembrane segments (Poolman and Konings, 1993).

In bacteria, transport systems may be either constitutively present or induced. While inducing transporters when required makes sense energetically, it is surprising that many transporters are constitutively present (Konings *et al.*, 1981). This feature is thought to provide a mechanism to manage any leaks of metabolic intermediates that passively diffuse from the cell. In addition, constitutive transporters may quicken the adaptation of a microorganism to changes in the external environment. Inducible transporters are also common and have been implicated in the transport of lactose in *E. coli* and citrate in *Bacillus subtilis* (Konings *et al.*, 1981).

1.D. Primary objectives

The first major objective of this research was to assess the degradative abilities of the psychrotrophic *P. fluorescens* with respect to ethylene glycol and propylene glycol. It was then investigated whether the microorganism could biodegrade both clean and spent Union Carbide UCAR deicing/anti-icing fluids at ambient temperatures.

The second major objective was to investigate the ethylene glycol transport mechanism. This information would serve to identify factors that could potentially interfere with ethylene glycol uptake. Variables investigated included pH, temperature, culture age and starvation. In addition, experiments were conducted to establish whether ethylene glycol uptake was energy dependent. The use of structurally similar compounds such as monohydric and dihydric alcohols as well as glycolate and diethylene glycol served to investigate the inducible nature of the transport mechanism as well as the specificity of this system.

2. Materials and Methods

2.A. General methods

2.A.1. Media preparation

For all experiments, the medium used was basal salts medium (1 L dH_2O, 4.3 g K_2HPO_4, 3.4 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 0.34 g $MgCl_2 \cdot 6H_2O$, 0.001 g $MnCl_2 \cdot 4H_2O$, 0.0006 g $FeSO_4 \cdot 7H_2O$, 0.026 g $CaCl_2 \cdot 2H_2O$, 0.002 g $NaMoO_4 \cdot 2H_2O$, adjusted to pH 7.0). For experiments conducted with ethylene glycol, propylene glycol (1,2-propanediol), glucose, glycolate, monohydric alcohols and Union Carbide UCAR deicing/anti-icing fluids, the basal salts medium (BSM) was prepared and autoclaved without the carbon source added. For sterilization of the carbon source, autoclaving was used for ethylene glycol, propylene glycol, glucose and the UCAR deicing/anti-icing fluids. To sterilize glycolate and the various monohydric alcohols, sterile Acrodisc® (Gelman Sciences, Ann Arbor, MI) filters (0.2 μm) were used. The carbon sources were then aseptically added to the sterile BSM. For experiments involving dihydric alcohols, the carbon source was added to the BSM prior to autoclaving. All experiments were conducted in 250-mL glass Erlenmeyer screw-top flasks with a culture volume of 50 mL. All reagents were purchased from either Sigma Chemical Co. (St. Louis, MO), BDH Inc. (Toronto, ON), Fisher Scientific (Nepean, ON) or Aldrich Chemical Co. (Milwaukee, WI), unless otherwise specified.

2.A.2. Maintenance of working culture

The microorganism used in this study was a psychrotrophic *Pseudomonas fluorescens* (Colucci and Inniss, 1996). The microorganism was maintained at both 25°C and 5°C using BSM supplemented with 1% (v/v) ethylene glycol (1% EG-BSM). Subculturing was performed when the culture reached the late exponential phase of growth. In addition, the culture was stored at -80°C (1.5-mL bacterial culture at late exponential phase and 0.3 mL sterile glycerol; pre-frozen in ethanol-dry ice prior to storage at -80°C).

2.A.3. Inoculum preparation

To prepare the inoculum for the experiments, it was first necessary to grow the culture in the appropriate medium. The culture was subcultured at least three times to ensure successful acclimation to the desired carbon source. At approximately mid-exponential phase, the cells were aseptically harvested by centrifugation (10,000 x g for 10 min) in a Sorvall RC2-B (Ivan Sorvall, Inc., Norwalk, CT) and the resulting pellet was washed three times with BSM (no carbon source added). The pellet was resuspended in a volume of BSM to provide an optical density (OD_{650}) of approximately 2.5. The inoculum was used to initiate the experiments; an OD_{650} of 0.05 was chosen as the initial target for all experiments.

2.A.4. Monitoring growth

To monitor growth of the microorganism, turbidity measurements were performed using a Pye Unicam SP8-100 spectrophotometer (Pye Unicam Ltd., Cambridge, England). Measurements were made at 650 nm.

2.A.5. Incubations for growth experiments

Fisher Scientific Isotemp incubators (Fisher Scientific, Nepean, ON) were used for both 25°C and 5°C experiments. The incubators contained shaker platforms that were set to 150 rpm. The temperature of the incubators was checked regularly with a YSI Tele-Thermometer (Model 42SC; Yellow Springs Instruments, Yellow Springs, OH). In addition, experiments at 5°C were sometimes done with a New Brunswick Gyrotory Water Bath Shaker (Model G67; New Brunswick Scientific, Edison, New Jersey) fitted to a Haake D1 coolant circulator (Haake, West Germany); the rotation of the shaker was 150 rpm.

2.A.6. Analysis of ethylene glycol and propylene glycol

Ethylene glycol and propylene glycol were analyzed by FID-gas chromatography (Shimadzu GC-9A, Shimadzu Corporation, Kyoto, Japan) using a Supelco Nukol column (15 m x 0.53 mm ID; Supelco, Bellefonte, PA) fitted with a 1-m precolumn (Supelco

Fused Silica Deactivated Tubing, 1 m x 0.53 mm ID; Supelco, Bellefonte, PA). A sample volume of 0.5 µL was used for each direct aqueous injection. Samples were injected in at least duplicate. The helium carrier gas, air and hydrogen were set to 20 mL/min, 480 mL/min and 36 mL/min, respectively. The injector and column were adjusted to 275°C and 120°C, respectively. A standard curve for each compound was prepared using the following concentrations: 5, 10, 25, 50 and 75 mg/L. The concentrations were prepared by diluting the appropriate compound in deionized water. To determine the lower detection limit of the analysis, the Method Detection Limit (MDL) was determined as defined in the report EPA-600/4-82-057.

2.A.7. Experimental design and statistics

All growth and utilization experiments were conducted in at least duplicate unless otherwise specified. The figures and tables present the averages and standard deviations calculated from these data.

2.B. Growth characteristics

2.B.1. Growth of *P. fluorescens* on ethylene glycol and propylene glycol at 25°C and 5°C

To test the growth of *P. fluorescens* on ethylene glycol and propylene glycol, flasks were prepared containing 10,000 mg/L of either substrate. To initiate the experiment, glucose acclimated cells were added. Samples were taken at various time points; growth was monitored by measuring both OD_{650} and viable cell counts. To determine the cell population, serial dilutions (10^{-2} to 10^{-7}) were prepared using BSM (absent of carbon source). Nutrient agar was used and each petri plate was divided into 3 equal sections; each section corresponded to a different dilution. A 10-µL sample was then taken from a dilution (i.e. 10^{-2}) and spotted upon the appropriate agar section. For each dilution, 5 spots were prepared. The plates were incubated at 25°C for 24 hours. Colonies were counted using a dissecting microscope (magnification = 8.75x; Vickers Instruments). For each dilution, the average of the five spots was used in the calculation of cell number.

From the resulting data, generation times were calculated using the exponential phase of growth.

2.C. Substrate utilization

2.C.1. Utilization of ethylene glycol and propylene glycol at 25°C and 5°C

Utilization of ethylene glycol and propylene glycol in BSM and a supplemented BSM (1 L dH_2O, 5.2 g K_2HPO_4, 4.1 g KH_2PO_4, 4.0 g $(NH_4)_2SO_4$, 0.34 g $MgCl_2 \cdot 6H_2O$, 0.001 g $MnCl_2 \cdot 4H_2O$, 0.0006 g $FeSO_4 \cdot 7H_2O$, 0.026 g $CaCl_2 \cdot 2H_2O$, 0.002 g $NaMoO_4 \cdot 2H_2O$, adjusted to pH 7.0) were compared both at 25°C and 5°C. Experiments were conducted in triplicate. Samples were taken at various time points; ethylene glycol and propylene glycol content was determined by gas chromatography (see section 2.A.6).

2.C.2. Changes in dissolved oxygen and pH during ethylene glycol utilization

To observe the changes which occurred in dissolved oxygen and pH of cultures during ethylene glycol utilization, a New Brunswick BioFlo C-30 Chemostat system (New Brunswick Scientific, Edison, New Jersey) was utilized. To record pH and dissolved oxygen content, a pH-21 pH Controller (New Brunswick Scientific, Edison, New Jersey) and DO-81 DO Controller (New Brunswick Scientific, Edison, New Jersey) units were used, respectively. Since the experiment was performed in batch mode, the overflow spout on the reaction vessel was plugged by a sterile rubber stopper. To the converted vessel, 300 mL of 1% EG-BSM was added. Aeration was set to 0.2 SLPM and agitation was set at 200 rpm. The vessel was inoculated with a culture of ethylene glycol acclimated cells to an initial OD_{650} of 0.05. Ethylene glycol concentration was determined using gas chromatography and growth was monitored by measuring OD_{650}. Replicate experiments were not conducted.

2.C.3. Utilization of a mixture of ethylene glycol and propylene glycol at 25°C and 5°C

To determine whether the presence of both ethylene glycol and propylene glycol had any effect upon the utilization of each component independently, a mixture of the

two glycols was prepared. For experiments conducted at 25°C, BSM was prepared containing 5,000 mg/L each of ethylene glycol and propylene glycol. At 5°C, BSM was prepared containing 10,000 mg/L each of ethylene glycol and propylene glycol. The flasks were inoculated with glucose acclimated cells. The concentration of ethylene glycol and propylene glycol was monitored by gas chromatography (see section 2.A.6) and growth was followed by measuring OD_{650}.

2.D. Union Carbide UCAR deicing/anti-icing fluid utilization

2.D.1. Growth of *P. fluorescens* on unused UCAR XL-54 and UCAR Ultra at 25°C

To determine whether growth was possible on unused UCAR deicing/anti-icing fluids (UCAR XL-54 and UCAR Ultra), various concentrations of each fluid were prepared in BSM. The concentrations chosen were: 10,000 mg/L, 25,000 mg/L, 50,000 mg/L, 75,000 mg/L and 100,000 mg/L (based on glycol). The flasks were inoculated with ethylene glycol acclimated cells; growth was monitored by measuring OD_{650}.

2.D.2. Utilization of unused UCAR XL-54 and UCAR Ultra at 25°C and 5°C

To investigate the ability of the psychrotroph to utilize unused deicing/anti-icing fluid, flasks were prepared which contained 10,000 mg/L of either UCAR XL-54 or UCAR Ultra. In addition, due to the exceptionally high biological oxygen demand of glycols, a nitrate supplemented media (standard BSM plus 10 mM $NaNO_3$) was also used in the event that the microorganism was capable of using nitrate as an alternate electron acceptor. The experiments were conducted at both 25°C and 5°C; the flasks were inoculated with ethylene glycol acclimated cells. Growth was monitored by measuring OD_{650} and ethylene glycol concentration was determined by gas chromatography.

2.D.3. Growth and utilization studies with spent UCAR deicing/anti-icing fluid at 25°C and 5°C

Spent UCAR deicing/anti-icing fluids were collected at Pearson International Airport Trillium Terminal 3 (Toronto, ON). The concentration of ethylene glycol in the spent fluids was measured by GC-FID and varied depending upon the deicing/anti-icing

activities prior to sampling. To determine whether *P. fluorescens* was capable of growth on the spent fluid, the fluid was diluted such that the ethylene glycol concentration was within a range that would permit growth, that is, 10,000 mg/L. Three diluents were used: BSM, groundwater and groundwater supplemented with 3.3 g/L K_2HPO_4, 3.4 g/L KH_2PO_4 and 2.0 g/L $(NH_4)_2SO_4$. The groundwater was obtained from a shallow sandy aquifer at CFB Borden (near Alliston, ON). Preliminary examination of the spent fluid had demonstrated the presence of naturally occurring microorganisms. To establish whether this population would contribute to ethylene glycol removal, the following three conditions were prepared:

1. nonsterile spent fluid / uninoculated
2. nonsterile spent fluid / inoculated with *P. fluorescens*
3. sterile spent fluid / inoculated with *P. fluorescens*

The entire experiment was conducted at both 25°C and 5°C. Inocula were pre-acclimated to ethylene glycol utilization at the appropriate temperature. Experiments conducted at 25°C and 5°C were performed in duplicate and triplicate, respectively. Growth was monitored by OD_{650} and ethylene glycol utilization was determined by gas chromatography.

2.D.4. Determination of the natural population in the spent fluid

In an effort to identify some of the microorganisms present in the spent fluid, isolates were prepared for analysis using a BioLog Microstation System (Biolog Inc., Hayward, CA). The spent fluid was diluted in BSM to an initial ethylene glycol concentration of 10,000 mg/L. Upon incubation at 25°C, growth appeared; from this growth, streak plates were prepared using Tryptic Soy Agar (TSA; Difco Laboratories, Detroit, MI). Gram stains were conducted on each isolate; isolates were restreaked on to fresh TSA and incubated for 12 h at 25°C. Each isolate was then processed according the directions defined in the BioLog Microstation System.

2.E. Oxygen consumption experiments – Determination of oxidase induction

2.E.1. Oxygen consumption protocol

Cells were grown on the carbon source corresponding to the conditions stipulated in the experimental design. Cells were harvested at approximately mid-exponential phase (OD_{650} of 0.8 to 1.2). To harvest, 1.5 mL of culture was transferred to a 1.5-mL Eppendorf tube and centrifuged at 12,000 x g for 15 s in an Eppendorf microcentrifuge (Model 5414; Brinkman Instruments, Rexdale, ON). The pellet was washed twice with BSM (carbon source free). The washed pellet was resuspended with BSM to an OD_{650} of 1.0 ($\pm$ 0.1).

The YSI Model 53 Oxygen Monitor (Yellow Springs Instruments, Yellow Springs, OH) was used to assess the oxygen consumed by the culture for each substrate tested. The protocol was as defined in the operating manual. Each reaction vessel received 2.5 mL of BSM (no carbon source added) and 0.5 mL of resuspended culture. Prior to the addition of the carbon source, the rate of oxygen consumption due to endogenous respiration was determined. To start the experiment, 30 µL of substrate was added to the reaction vessel using a syringe. The results of the experiment were recorded using a BBC SE120 chart recorder over a 20 minute period. Oxygen consumption for the selected substrate was adjusted for endogenous respiration.

2.E.2. Substrates tested

The aim behind the oxygen consumption tests was to establish whether the initial steps of ethylene glycol and propylene glycol metabolism were mediated by the same primary alcohol oxidase. To test this, cells were first grown on ethylene glycol and subsequently tested for oxidase activity when presented with ethylene glycol as a growth substrate. Similarly, propylene glycol grown cells were tested for the ability to separately oxidize ethylene glycol and propylene glycol. If the oxidase in question was specific and induced only upon acclimation to one of the two substrates, different rates of oxidation would be noted. Conversely, strong oxidation of both substrates in propylene glycol

grown cells would suggest that the oxidase is the same in both pathways and was thus induced by both ethylene glycol and propylene glycol.

2.F. Uptake studies involving ^{14}C-ethylene glycol

2.F.1. General uptake protocol

Cells were grown on the carbon source corresponding to the conditions stipulated in the experimental design. To prepare the cells for uptake studies, cultures were harvested at approximately mid-exponential phase (OD_{650} of 0.8 to 1.2). To harvest, the cells were transferred to 50-mL polypropylene centrifuge tubes and centrifuged at 10,000 x g for 10 min at 4°C in a Sorvall RC2-B centrifuge. The cells were washed twice and then resuspended in BSM (without carbon source) to an OD_{650} of 1.0 (± 0.1). The experiments were done in 18 mm x 150 mm glass test tubes. Constant temperature was maintained by incubating the reaction tubes in a 1-L beaker fitted with cooling/heating coils attached to a Haake D1 circulator. To ensure mixing of the tube contents, micro stir bars were placed in each tube and the 1-L beaker was placed on top of a stir plate. In addition to live cells, a boiled cell preparation was also tested to investigate whether nonspecific binding of ^{14}C-ethylene glycol was occurring. To prepare the killed cells, a sample of washed cells (~10 mL) was transferred to an 18-mm x 150-mm test tube and placed into a boiling waterbath for 5 minutes. The cells were cooled and then used for uptake studies at 25°C.

Each reaction mixture contained 2 mL of washed cells, 2 mL of 50 mM phosphate buffer and 17 µL of 0.11 M chloroamphenicol (34 mg chloramphenicol in 1 mL ethanol). The mixture was allowed to equilibrate for 15 min. To start the reaction, ^{14}C-ethylene glycol (Sigma Chemical Company, St. Louis, MO) was added to a final concentration of 66.7 µM at a specific activity of 1.5 µCi/µmol. In substrate competition studies, the alternate substrate was added at the same time as the ^{14}C-ethylene glycol. After addition of ^{14}C-ethylene glycol, 0.5-mL samples were taken at 15-min intervals for 45 min. The samples were placed in 1.5-mL Eppendorf tubes and centrifuged at 12,000 x g for 2 min

in an Eppendorf microcentrifuge (Model 5414). The supernatant was removed and discarded; the pellet was resuspended in 0.6 mL 50 mM phosphate buffer (pH 7.2). To measure the uptake of radioactivity, 0.5 mL of the resuspended pellet was filtered through a Whatman GF/C filter (24 mm; VWR Canlab, Mississauga, ON). To minimize binding of the ethylene glycol to the glass microfibre filter, the filter was presoaked in 50 mM phosphate buffer containing 10,000 mg/L ethylene glycol. After the application of 0.5 mL of resuspended pellet to the filter, the sample was washed with 10 mL of 50 mM phosphate buffer containing 10,000 mg/L ethylene glycol. The filter was then removed from the filter apparatus and placed into a scintillation vial to which 10 mL of Ready Safe liquid scintillation cocktail (Beckman Instruments Inc., Fullerton, CA) was added. The level of radioactivity was measured 24 h after the addition of the scintillation cocktail using a Beckman LS7000 liquid scintillation spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

All uptake studies were conducted in at least duplicate. The figures and tables present the averages and standard deviations from these data.

2.F.2. Effect of culture age upon ethylene glycol uptake

To determine the effects of culture age upon ethylene glycol uptake, cells acclimated to ethylene glycol utilization at 25°C were used. To harvest the cells without utilizing the entire culture, 6 samples (1.5 mL each) were withdrawn and placed into 1.5-mL Eppendorf tubes. To obtain a representative range of culture ages, samples were withdrawn at early-exponential, mid-exponential, late-exponential phase (2 different times) and decline phase. The samples were centrifuged (12,000 x g; Eppendorf Centrifuge 5414) and washed twice with BSM (without carbon source). In this instance, the washed pellet was resuspended in BSM (carbon source free) to an OD_{650} of approximately 0.65; the remainder of the procedure was as described in section 2.F.1.

2.F.3. Induction response of the ethylene glycol uptake mechanism

The uptake of ethylene glycol may be an inducible process. To test this, cells were acclimated to glucose or glycolate prior to harvesting. The cells were washed, prepared for use, and tested for ^{14}C-ethylene glycol uptake as described in section 2.F.1.

2.F.4. Effect of transport inhibitors upon ethylene glycol uptake

To test the effects of transport inhibitors upon ethylene glycol uptake, cells acclimated to ethylene glycol utilization at 25°C were used. The inhibitors chosen for these experiments were 2,4-dinitrophenol (DNP; an uncoupler and inhibitor of the proton-motive force; Sigma Chemical Co., St. Louis, MO) and N,N'-dicyclohexylcarbodiimide (DCCD; an inhibitor of membrane-bound ATPase; Sigma Chemical Co., St. Louis, MO). The DNP and DCCD were prepared as 100 mM stock solutions in N,N-dimethylformamide (DMFA; Caledon Laboratories, Georgetown, ON). For each inhibitor, 0.1 mM and 1 mM concentrations were used. The inhibitor was added at the beginning of the 15 min equilibration period prior to the addition of the ^{14}C-ethylene glycol. The protocol was as described in section 2.F.1.

2.F.5. Effect of pH upon ethylene glycol uptake

Cells were acclimatized to ethylene glycol utilization at 25°C in standard BSM (pH 7). To test the effects of pH, ethylene glycol uptake was tested at pH 4, pH 5, pH 6, pH 7 and pH 8. The various pH solutions were based on BSM (pH adjusted with H_2SO_4 or NaOH). The protocol described in section 2.F.1 was used to test the effects of pH upon ^{14}C-ethylene glycol uptake.

2.F.6. Effect of temperature upon ethylene glycol uptake

Cells were acclimatized to ethylene glycol utilization at 25°C in standard BSM (pH 7). To test the effects of temperature, the following temperatures were chosen: 0, 5, 10, 25, 30°C. The temperatures were maintained by a Haake D1 coolant circulator. The protocol described in section 2.F.1 was used to test the effects of temperature upon ^{14}C-ethylene glycol uptake.

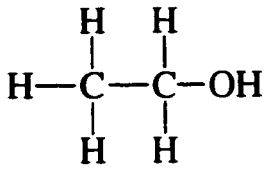
2.F.7. Effect of monohydric alcohols upon ethylene glycol uptake

Monohydric alcohols have structural similarities with ethylene glycol. By investigating the effects of various monohydric alcohols upon ethylene glycol uptake, information regarding the nature of the transport mechanism can be obtained. The monohydric alcohols selected for investigation were ethanol, 1-propanol, 2-propanol, 1-butanol and 2-butanol (Fig. 3). The first step in working with the monohydric alcohols was to determine which supported growth of *P. fluorescens*. The concentrations of the alcohols in the media were such that equimolar concentrations of carbon would be present. Therefore, the concentrations used were as follows: 0.05 M ethanol, 0.033 M 1-propanol, 0.033 M 2-propanol, 0.025 M 1-butanol and 0.025 M 2-butanol. The alcohols were sterilized separately from the BSM using sterile 0.2 μm Acrodisc® filters (Gelman Sciences, Ann Arbor, MI). The alcohol supplemented medium was then inoculated with washed cells which had been acclimated to ethylene glycol utilization at 25°C. The flasks were incubated at 25°C and growth curves were determined.

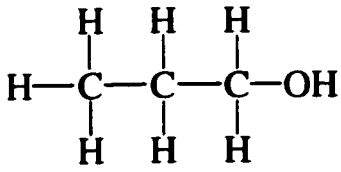
For each alcohol that supported growth, acclimated cultures were then prepared and the uptake protocol (described in section 2.F.1) was followed to test the ^{14}C-ethylene glycol uptake potential.

Another series of experiments investigated the ability of monohydric alcohols to compete with ^{14}C-ethylene glycol for the transport mechanism. In these experiments, cells acclimated to ethylene glycol utilization at 25°C were prepared and tested as outlined in section 2.F.1. However, when the ^{14}C-ethylene glycol was added, one of the monohydric alcohols was also added. For each alcohol tested, the concentrations were as follows: 0.05 M ethanol, 0.033 M 1-propanol, 0.033 M 2-propanol, 0.025 M 1-butanol and 0.025 M 2-butanol. The presence of one of the monohydric alcohols would serve as a competitor to the ^{14}C-ethylene glycol in the event that the transport mechanism was compatible with that particular alcohol.

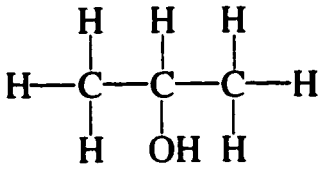
Figure 3: Monohydric alcohols used in ^{14}C-ethylene glycol uptake experiments.



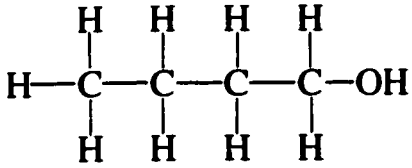
Ethanol



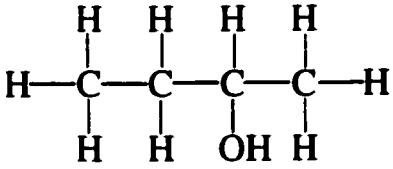
1-Propanol



2-Propanol



1-Butanol



2-Butanol

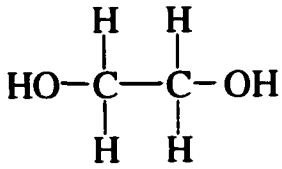
2.F.8. Effect of dihydric alcohols upon ethylene glycol uptake

Like monohydric alcohols, dihydric alcohols have structural similarities with ethylene glycol. By investigating the effects of various dihydric alcohols upon ethylene glycol uptake, information regarding the nature of the transport mechanism can be obtained. The dihydric alcohols selected for investigation were 1,2-ethanediol (ethylene glycol), 1,2-propanediol (propylene glycol), 1,3-propanediol, 1,2-butanediol, 1,3-butanediol, 1,4-butanediol, 2,3-butanediol, 1,2-pentanediol, 1,5-pentanediol, 2,4-pentanediol, 1,2-hexanediol (Fig. 4). Before conducting any uptake experiments with the dihydric alcohols, it was necessary to determine which supported growth of *P. fluorescens*. The concentrations of the dihydric alcohols in the media were such that equimolar concentrations of carbon would be present. Therefore, the concentrations used were as follows: 0.18 M 1,2-ethanediol, 0.12 M 1,2-propanediol, 0.12 M 1,3-propanediol, 0.09 M 1,2-butanediol, 0.09 M 1,3-butanediol, 0.09 M 1,4-butanediol, 0.09 M 2,3-butanediol, 0.07 M 1,2-pentanediol, 0.07 M 1,5-pentanediol, 0.07 M 2,4-pentanediol, 0.06 M 1,2-hexanediol. The autoclaved supplemented medium was then inoculated using washed cells which had been acclimated to ethylene glycol utilization at 25°C. The flasks were incubated at 25°C and sampled at regular time points such that a growth curve could be generated for each medium type. Replicates were not conducted for the growth experiments.

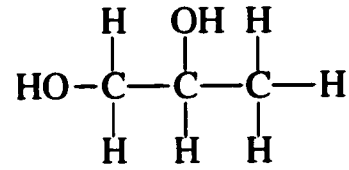
To prepare for the experiment, cells were acclimated to each dihydric alcohol which supported growth of the microorganism. Each of these cultures was subcultured at least three times to ensure acclimation to the target substrate. The protocol to test the uptake potential of alcohol acclimated cells was as described in section 2.F.1.

In addition to testing for the induction of ethylene glycol transport with dihydric alcohol acclimated cells, a series of experiments investigating the ability of dihydric alcohols to compete with ^{14}C-ethylene glycol for the transport mechanism was performed. In these experiments, cells acclimated to ethylene glycol utilization at 25°C

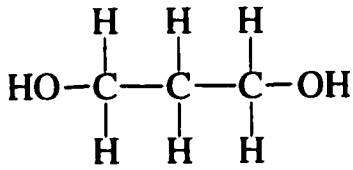
Figure 4: Dihydric alcohols used in ^{14}C-ethylene glycol uptake experiments.



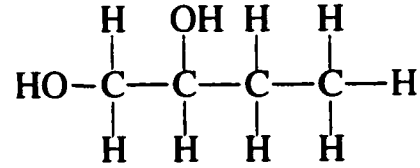
1,2-Ethanediol



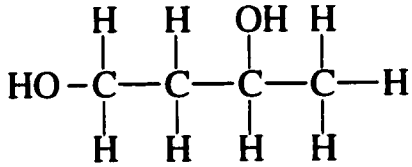
1,2-Propanediol



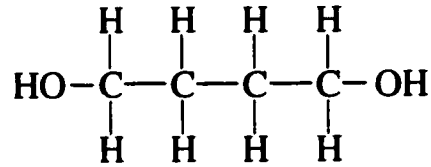
1,3-Propanediol



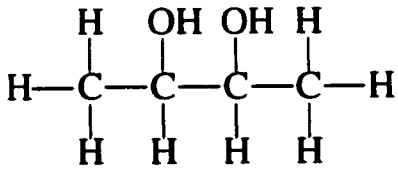
1,2-Butanediol



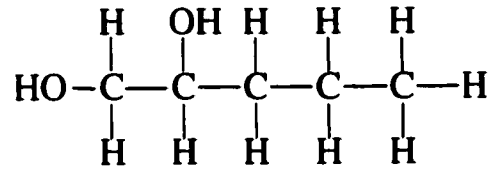
1,3-Butanediol



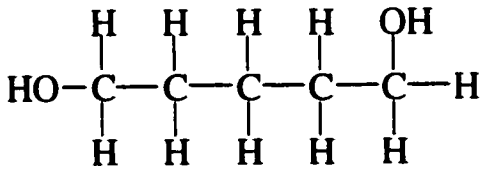
1,4-Butanediol



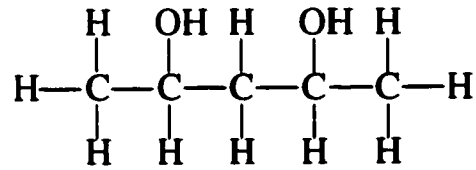
2,3-Butanediol



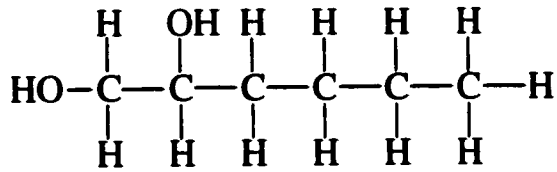
1,2-Pentanediol



1,5-Pentanediol



2,4-Pentanediol



1,2-Hexanediol

were prepared and tested as outlined in section 2.F.1. However, when the ^{14}C-ethylene glycol was added, one of the dihydric alcohols was also added. For each dihydric alcohol tested, the concentration used was the same as that used to test for growth. The dihydric alcohol would serve as a competitor to the ^{14}C-ethylene glycol in the event that the transport mechanism is compatible with that particular alcohol.

2.F.9. Effect of diethylene glycol upon ethylene glycol uptake

To test the effects of diethylene glycol upon ethylene glycol uptake, cells acclimated to ethylene glycol utilization at 25°C were used. The experiments were designed as outlined in section 2.F.7 and 2.F.8 where monohydric and dihydric alcohols, respectively, were tested for their ability to compete with ethylene glycol for the transport process. The diethylene glycol was added to the reaction vessel such that the molar concentration of carbon was equivalent to that found in BSM supplemented with 10,000 mg/L ethylene glycol.

2.F.10. Effect of starvation upon ethylene glycol uptake

To determine the effects of starvation upon ethylene glycol uptake, cells acclimated to ethylene glycol utilization at 25°C were used. When the cultures reached mid-exponential, they were harvested and washed as explained in section 2.F.1. The washed pellet was then resuspended in 50 mL BSM (without carbon source) in a 250-mL screw-top Erlenmeyer flask. The flask was incubated at 25°C and samples were taken at 0 day, 1 day, 7 days and 15 days. The samples were processed and tested as outlined in section 2.F.1.

3. Results

3.A. General methods

3.A.1. Ethylene glycol analysis

Ethylene glycol analysis was conducted using a Shimadzu GC-9A gas chromatograph fitted with a Supelco Nukol column (15m x 0.53mm ID). Standard solutions representing 5, 10, 25, 50, and 75 mg/L were used to prepare a standard curve (Fig. 5). The values plotted represent an average of 3 injections and the points were fitted using linear regression. The method detection limit (MDL) was determined to be 3.2 mg/L.

3.A.2. Propylene glycol analysis

Propylene glycol analysis was conducted using a Shimadzu GC-9A gas chromatograph fitted with a Supelco Nukol column (15m x 0.53mm ID). Standard solutions representing 5, 10, 25, 50 and 75 mg/L were used to prepare a standard curve (Fig. 6). The values plotted represent an average of 3 injections and the points were fitted using linear regression. The method detection limit (MDL) was determined to be 4.2 mg/L.

3.B. Growth Characteristics

3.B.1. Growth of *P. fluorescens* on ethylene glycol and propylene glycol at 25°C and 5°C

The psychrotrophic *P. fluorescens* used in this study was able to grow on ethylene glycol, as measured by OD_{650} and viable cell counts; growth on propylene glycol was also shown to occur (Fig. 7 and Fig. 8). At 25°C, growth on propylene glycol and ethylene glycol was similar with generation times of 7.2 h and 7.5 h, respectively. This similarity was also observed at 5°C where propylene glycol grown cells exhibited a generation time of 49.0 h compared to 56.6 h for ethylene glycol grown cells. In Figure 8, it is evident that acclimation to 5°C was slower with propylene glycol grown cells;

Figure 5: Standard curve for the analysis of ethylene glycol on the Shimadzu GC-9A. The method detection limit was 3.2 mg/L.

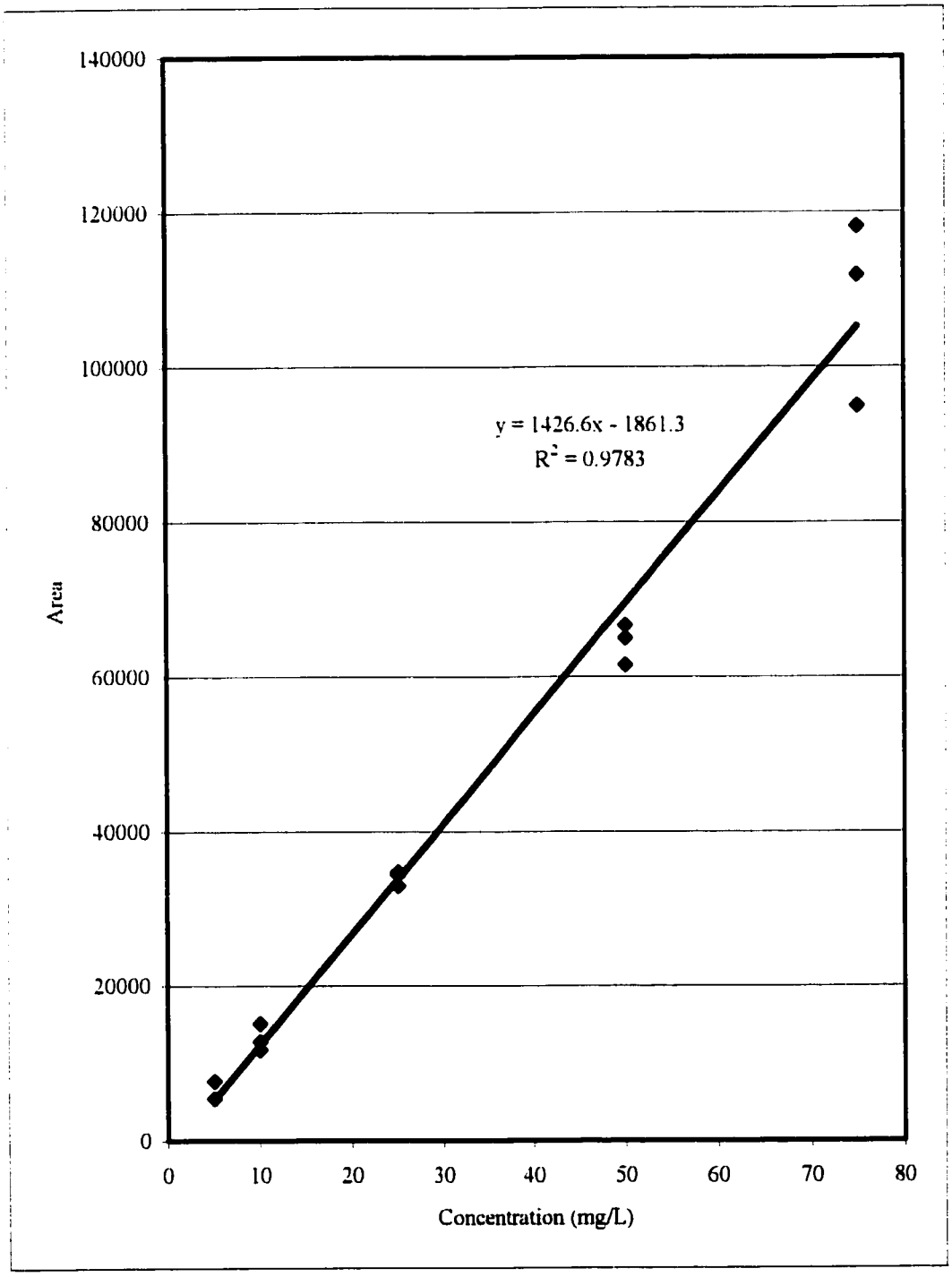

140000
120000
100000
80000
60000
40000
20000
0
Area
y = 1426.6x - 1861.3
R² = 0.9783
0
10
20
30
40
50
60
70
80
Concentration (mg/L)

Figure 6: Standard curve for the analysis of propylene glycol on the Shimadzu GC-9A. The method detection limit was 4.2 mg/L.

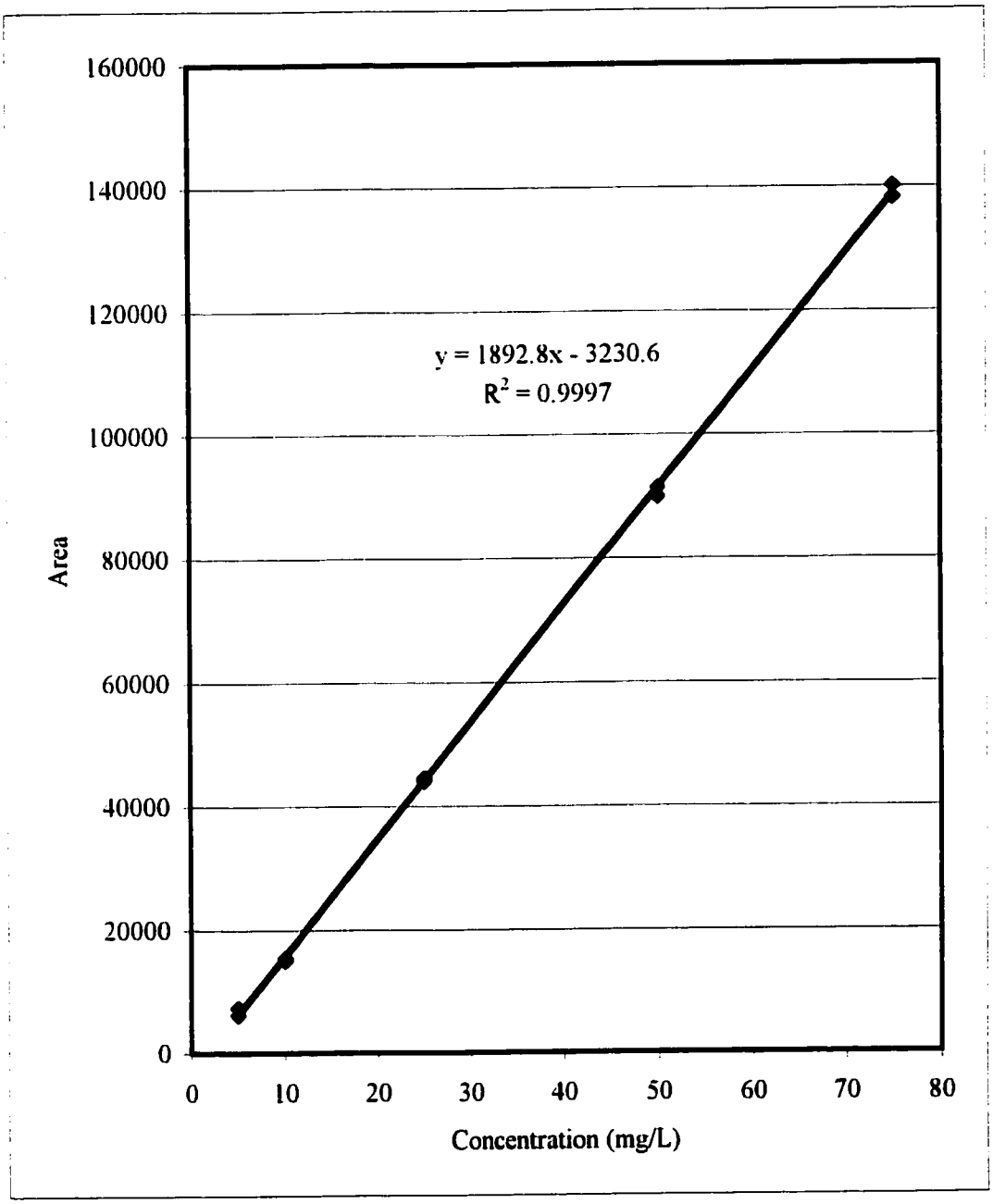
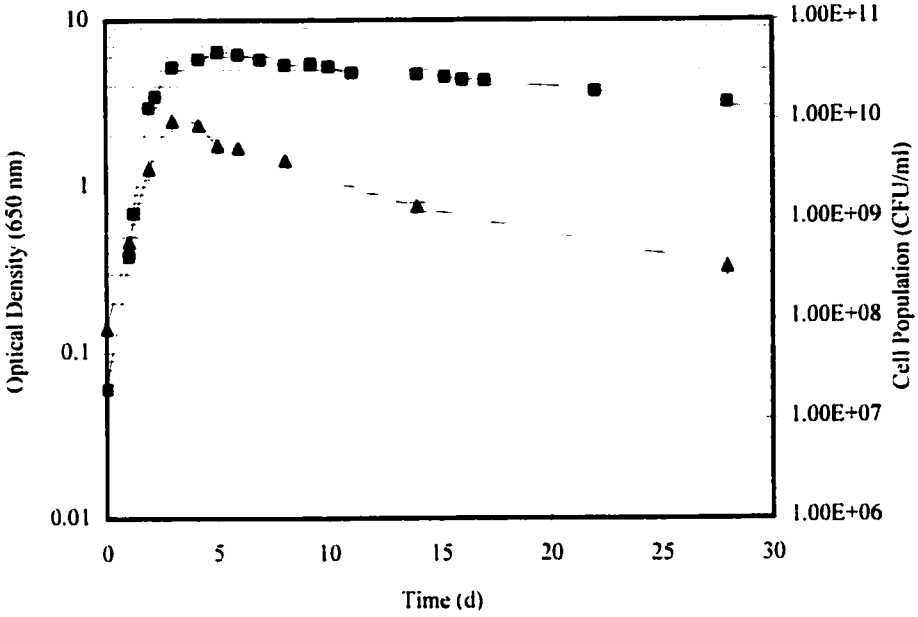

160000
140000
120000
100000
80000
60000
40000
20000
0
Area
y = 1892.8x - 3230.6
R2 = 0.9997
0
10
20
30
40
50
60
70
80
Concentration (mg/L)

Figure 7: Growth of *P. fluorescens* on ethylene glycol and propylene glycol at 25°C. Growth was carried out in basal salt media with an initial glycol concentration of 10,000 mg/L. Optical density was measured spectrophotometrically and cell population was determined using viable cell counts. (OD_{650} (■), viable cell count (▲))

Ethylene Glycol



Propylene Glycol

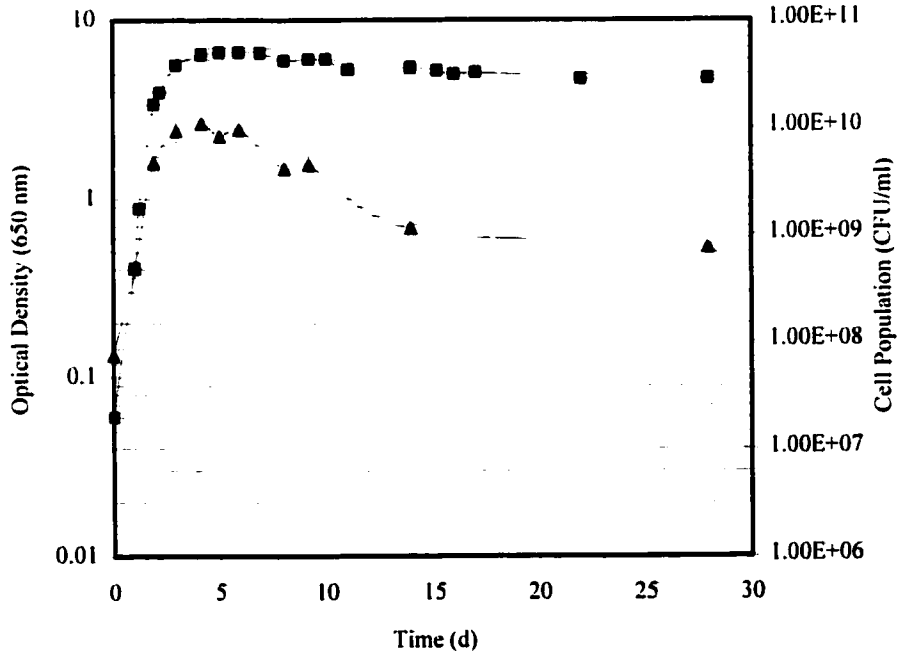
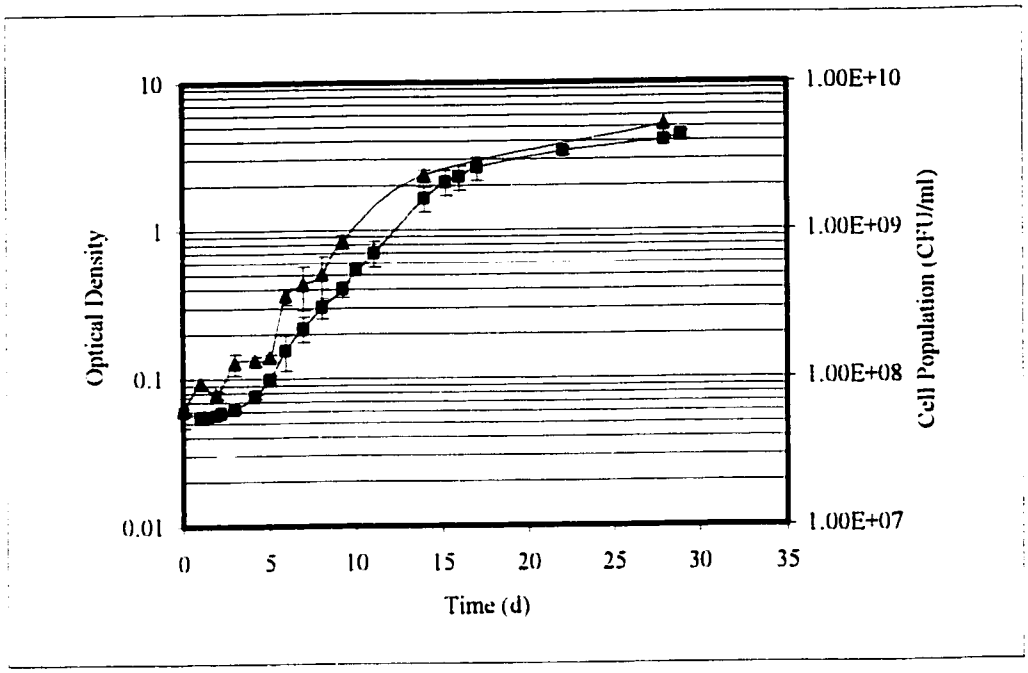
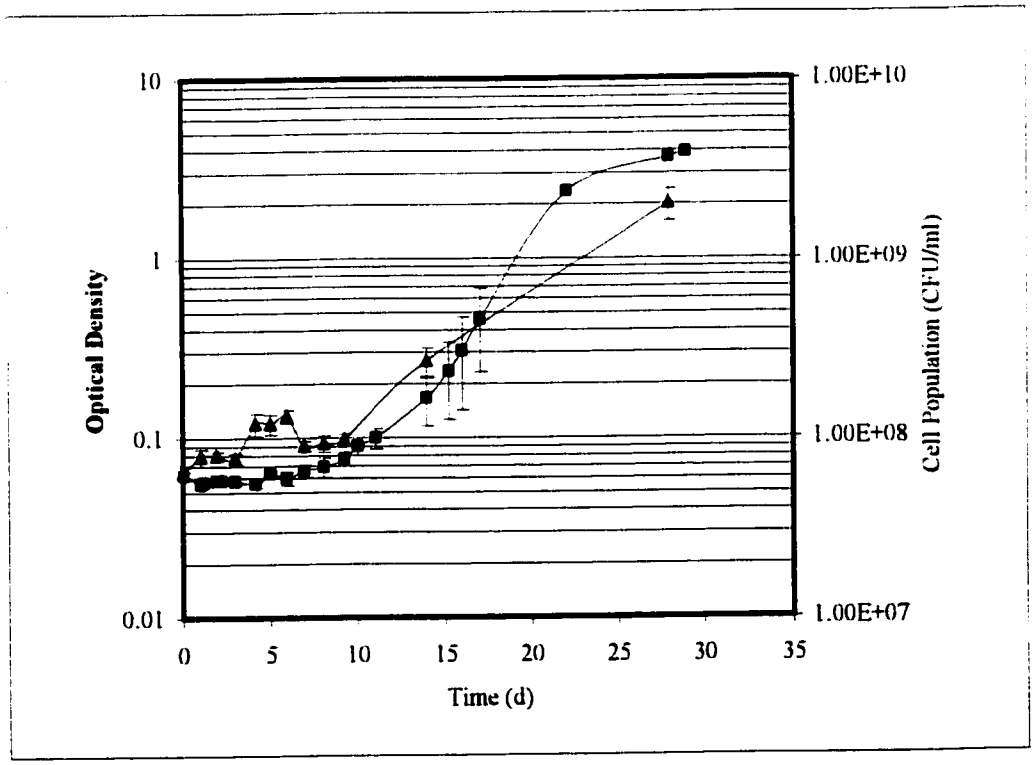


Figure 8: Growth of *P. fluorescens* on ethylene glycol and propylene glycol at 5°C. Growth was carried out in basal salt media with an initial glycol concentration of 10,000 mg/L. Optical density was measured spectrophotometrically and cell population was determined using viable cell counts. (OD_{650} (■), viable cell count (▲))

Ethylene Glycol



Propylene Glycol



ethylene glycol grown cells acclimatized to the lower temperature in approximately 5 days whereas propylene glycol grown cells required approximately 10 days.

3.C. Ethylene glycol and propylene glycol utilization

3.C.1. Utilization of ethylene glycol and propylene glycol at 25°C and 5°C

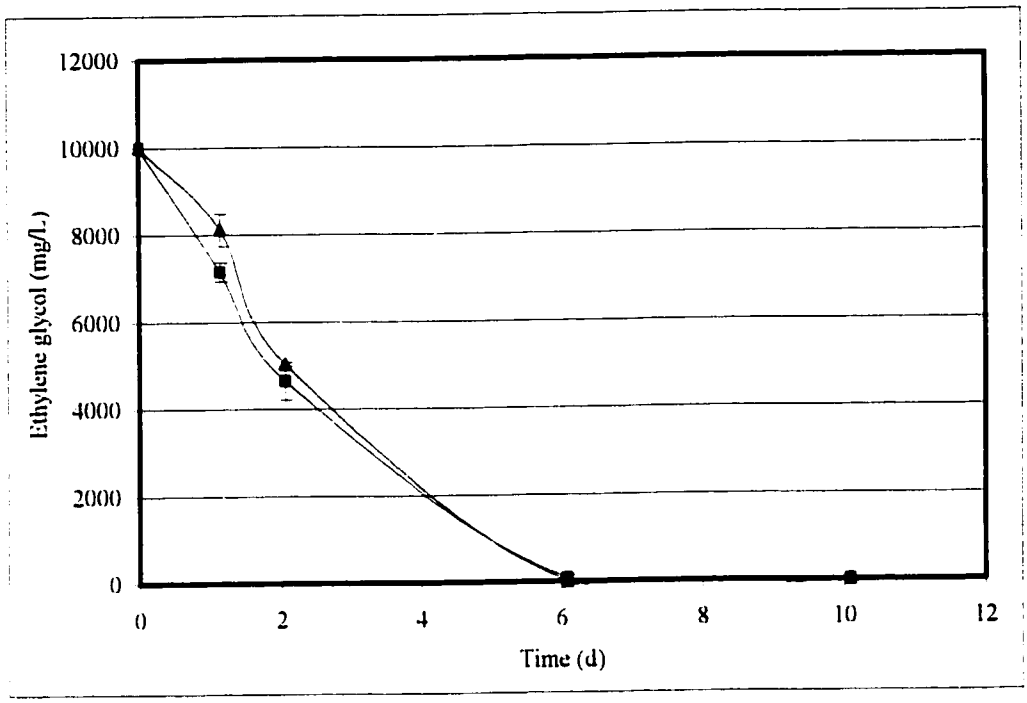
The removal of ethylene glycol and propylene glycol at 25°C and 5°C is illustrated in Figures 9 and 10 and summarized in Table 1. In Figures 7 and 8, it was evident that growth on these substrates resulted in high optical densities; it was therefore of interest to determine whether an enhanced BSM (20% increase in K_2HPO_4 and KH_2PO_4, 100% increase in $(NH_4)_2SO_4$) would affect the rates of utilization. The results using the enhanced media are also presented in Figures 9 and 10 and summarized in Table 1. The initial rates of utilization were similar for ethylene glycol and propylene glycol, however, complete removal of the substrate was quite different. At 25°C, greater than 99% removal of ethylene glycol and propylene glycol occurred at approximately 6 days and 35 days, respectively. A similar trend was observed at 5°C where greater than 99% removal of ethylene glycol and propylene glycol occurred after approximately 36 days and 90 days, respectively. Furthermore, the use of supplemented media did not offer any significant benefit with respect to glycol removal when compared to standard BSM. For all further studies, standard BSM was used.

3.C.2. Changes in dissolved oxygen and pH during ethylene glycol utilization at 25°C

When ethylene glycol undergoes aerobic biodegradation, a large quantity of oxygen is consumed. If the use of available dissolved oxygen exceeds that of natural replenishment, aquatic plants and animals can be negatively affected. In addition, microorganisms may also be affected by changes in the environment when ethylene glycol is degraded. To determine how the environment changes in batch culture, the dissolved oxygen, pH, ethylene glycol concentration and OD_{650} were monitored.

Figure 9: Utilization of ethylene glycol by *P. fluorescens* at 25°C and 5°C. In addition to standard basal salts medium, a supplemented medium containing increased nitrogen and phosphorus levels was implemented. Glycol removal was measured using gas chromatography. (Standard BSM (■),Supplemented BSM (▲))

25°C


12000
10000
8000
6000
4000
2000
0
Ethylene glycol (mg/L)
0
2
4
6
8
10
12
Time (d)

5°C

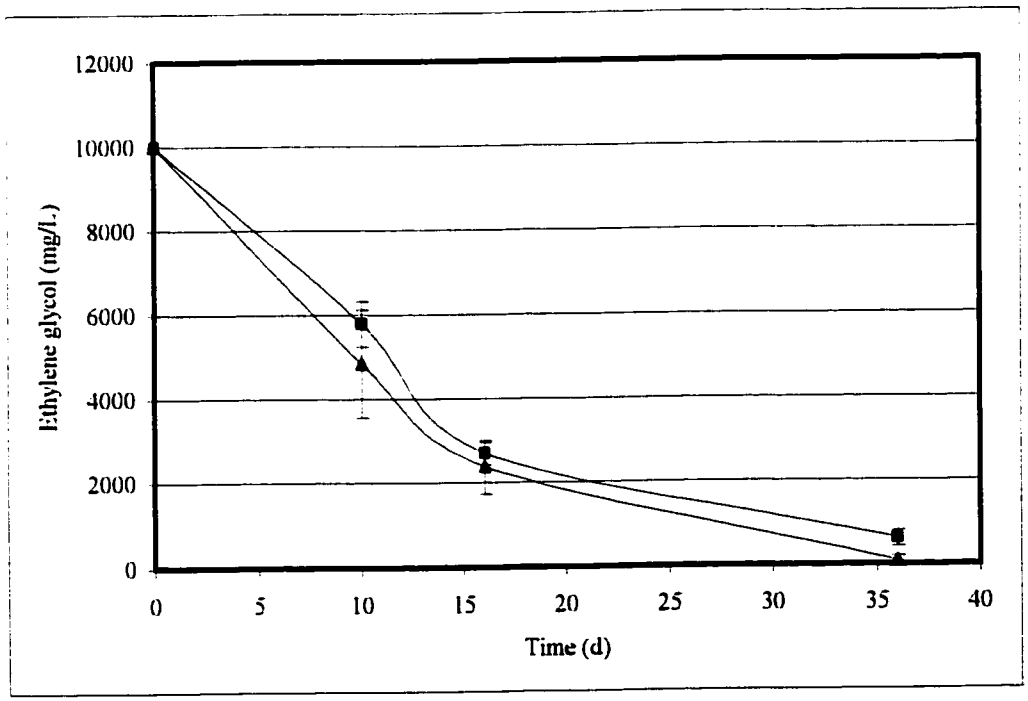
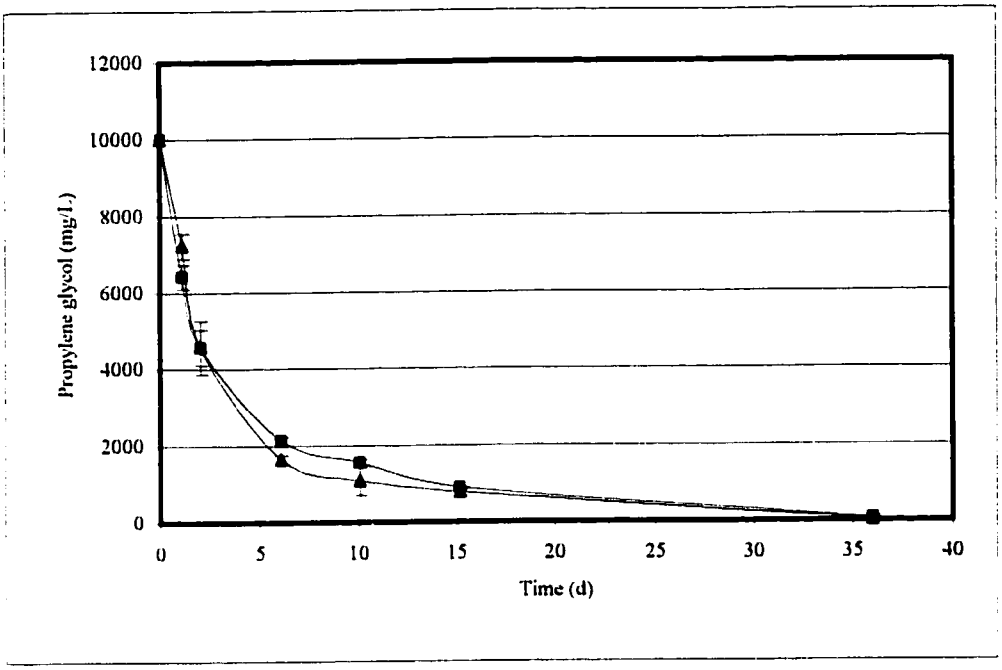

12000
10000
8000
6000
4000
2000
0
Ethylene glycol (mg/L)
0
5
10
15
20
25
30
35
40
Time (d)

Figure 10: Utilization of propylene glycol by *P. fluorescens* at 25°C and 5°C. In addition to standard basal salts medium, a supplemented medium containing increased nitrogen and phosphorus levels was implemented. Glycol removal was measured using gas chromatography. (Standard BSM (■), Supplemented BSM (▲))

25°C


12000
10000
8000
6000
4000
2000
0
Propylene glycol (mg/L)
0
5
10
15
20
25
30
35
40
Time (d)

5°C

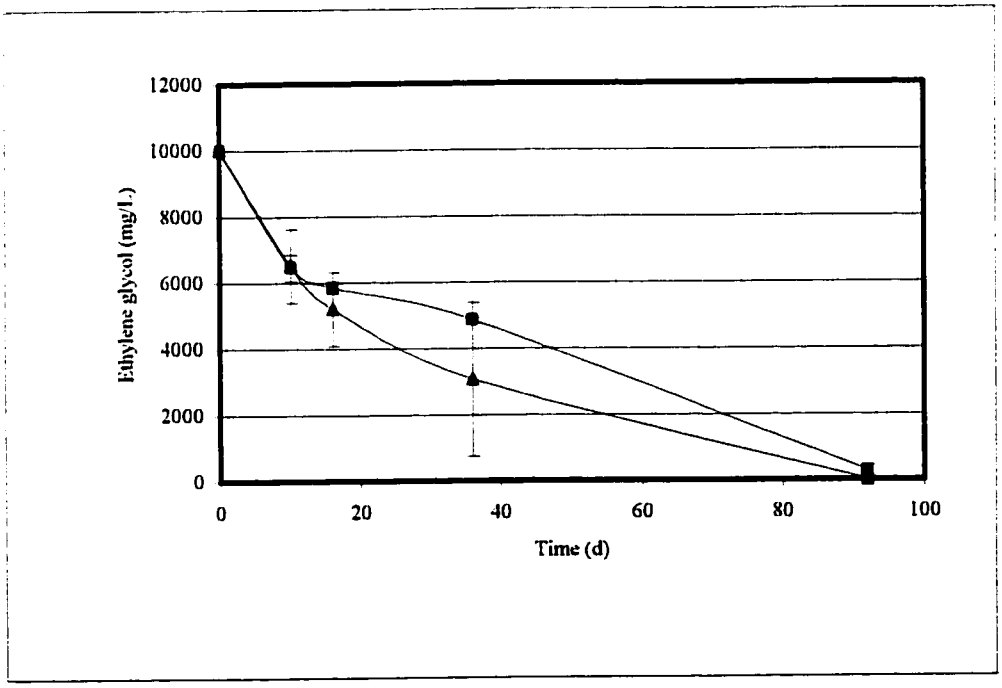

12000
10000
8000
6000
4000
2000
0
Ethylene glycol (mg/L)
0
20
40
60
80
100
Time (d)

Table 1: Initial utilization of ethylene glycol and propylene glycol at 25°C and 5°C.

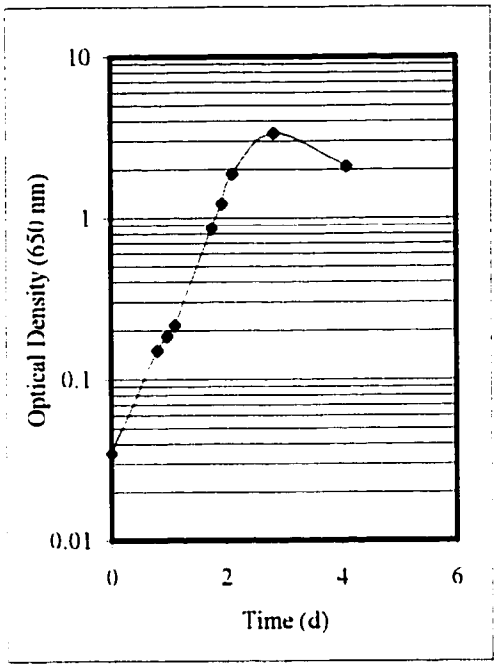
Substrate/Condition	Utilization (mg/L/day)	
Ethylene glycol	25°C	5°C
Standard Media	2596 ± 201	422 ± 53
Enhanced Media	2390 ± 38	516 ± 128
Propylene Glycol	25°C	5°C
Standard Media	2650 ± 211	355 ± 40
Enhanced Media	2441 ± 337	349 ± 111

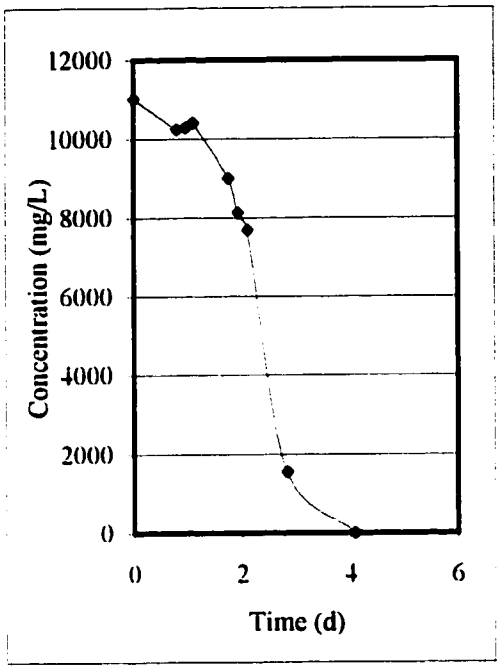
Ethylene glycol utilization led to a reduction in the dissolved oxygen concentration even while the vessel was being aerated (Fig. 11). Once degradation was complete, the dissolved oxygen returned to approximately 100%. The pH of the culture was also affected during batch growth and it is interesting to note that the pH profile generated closely matched that of ethylene glycol removal. Upon complete removal, the pH of the vessel was 5.9.

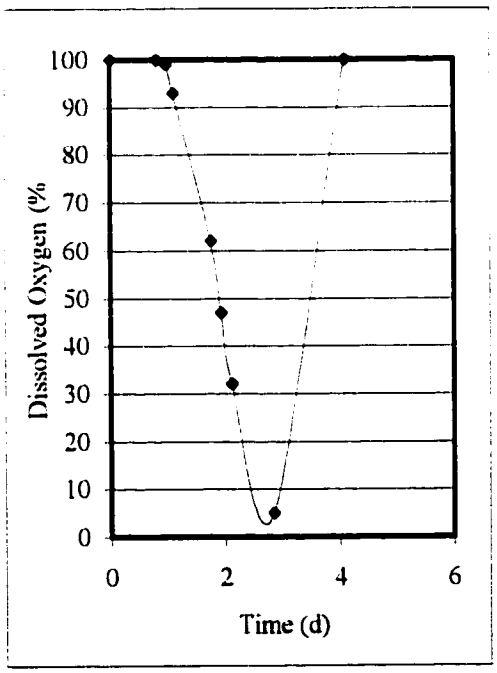
3.C.3. Utilization of a mixture of ethylene glycol and propylene glycol at 25°C and 5°C

Both ethylene glycol and propylene glycol are commonly used in deicing/anti-icing fluids. As a result, it is possible that during the treatment of spent fluids, both of these glycols may be present together. Thus it was determined how *P. fluorescens* would function where both glycols were present at equal concentrations. The experiment was conducted at both 25°C and 5°C (Fig. 12 and Table 2). At 25°C, the generation time for the combined substrates was 6.8 h, which was very similar to the generation times for the individual substrates (3.B.1). At 5°C, the generation time of the combined substrates was 55.4 h, which was also very similar to the generation times obtained on the individual substrates (3.B.1). The ethylene glycol and propylene glycol were utilized concurrently. The initial rates of ethylene glycol and propylene glycol utilization at 25°C were 1661 and 750 mg/L/day, respectively. For 5°C, the initial rates of ethylene glycol and propylene glycol utilization were 342 and 139 mg/L/day, respectively. What is interesting to note is that for each temperature, the rates observed added up to a utilization rate that was similar to experiments in which only one substrate was used (see Table 2). For example, at 25°C, the combined rate of utilization for glycols was 2411 mg/L/day (1661 + 750 mg/L/day) and this was quite comparable to the individual rates for ethylene glycol and propylene glycol which were 2596 and 2650 mg/L/day, respectively. In Figure 12, it is evident that complete degradation did not occur. The

Figure 11: Changes in dissolved oxygen and pH during ethylene glycol utilization at 25°C. Growth was carried out in batch mode in a New Brunswick BioFlo C-30 Chemostat. The pH and dissolved oxygen were measured using the pH-21 pH controller and DO-81 DO controller, respectively. Growth was monitored spectrophotometrically and glycol content was determined by gas chromatography. (The data points plotted represent single values)


Optical Density (650 nm)
10
1
0.1
0.01
0
2
4
6
Time (d)


Concentration (mg/L)
12000
10000
8000
6000
4000
2000
0
0
2
4
6
Time (d)


Dissolved Oxygen (%
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
Time (d)

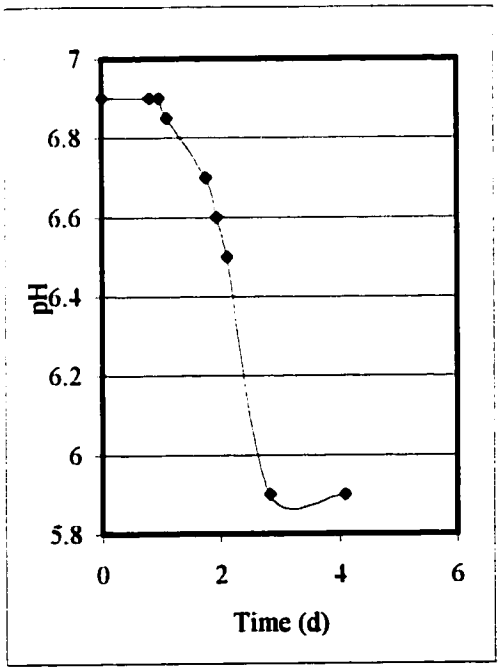
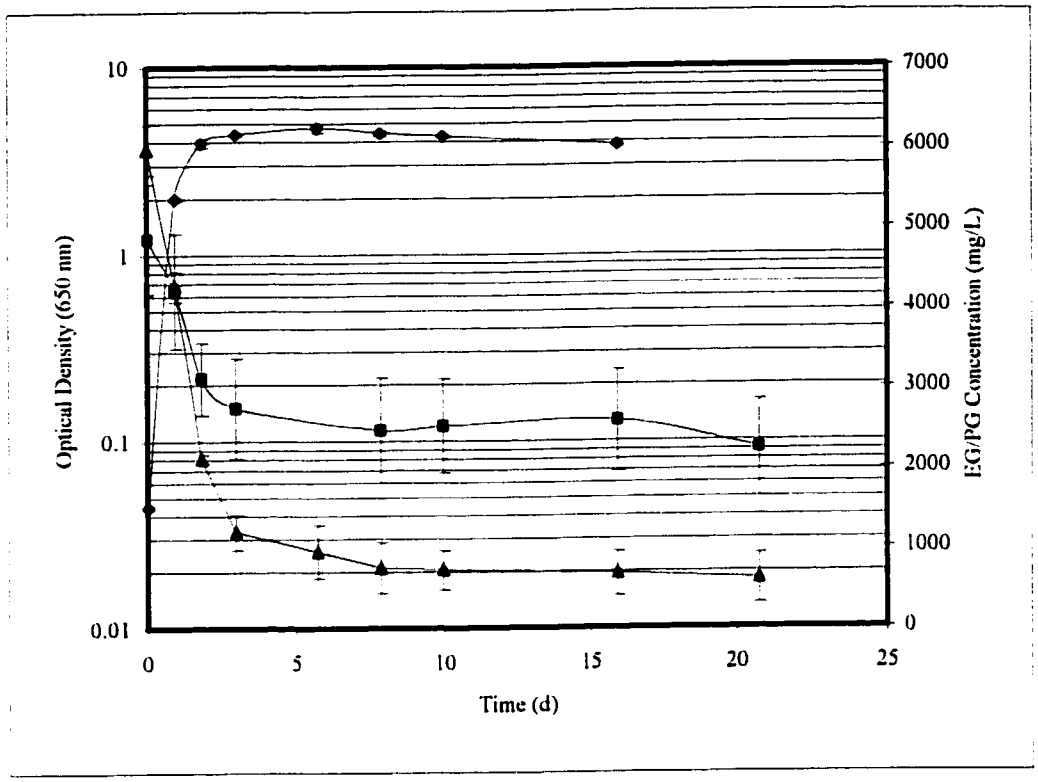

pH
7
6.8
6.6
6.4
6.2
6
5.8
0
2
4
6
Time (d)

Figure 12: Growth and utilization of a mixture of ethylene glycol and propylene glycol at 25°C and 5°C. Growth was monitored spectrophotometrically and glycol removal was determined using gas chromatography. (optical density (◆), propylene glycol (■), ethylene glycol (▲))

25°C


Optical Density (650 nm)
10
1
0.1
0.01
0
5
10
15
20
25
Time (d)
7000
6000
5000
4000
3000
2000
1000
0
EG/PG Concentration (mg/L)

5°C

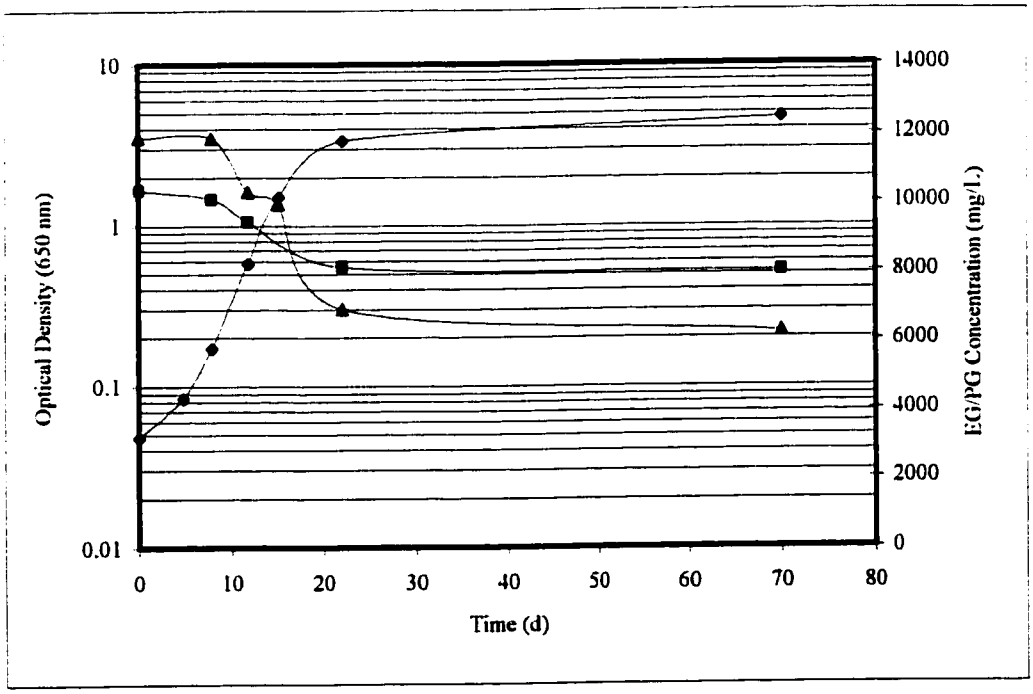

Optical Density (650 nm)
10
1
0.1
0.01
0
10
20
30
40
50
60
70
80
Time (d)
14000
12000
10000
8000
6000
4000
2000
0
EG/PG Concentration (mg/L)

Table 2: Growth and utilization of a mixture of ethylene glycol and propylene glycol at 25°C and 5°C.

Substrate/Condition	Generation Time (h)	
EG/PG 25°C	6.8 ± 0.0	
EG/PG 5°C	55.4	
Substrate/Condition	**Utilization (mg/L/day)**	
	25°C	5°C
Ethylene Glycol	1661 ± 37	342.3
Propylene Glycol	750 ± 44	138.84

reason for such an observation is unclear but it may be related to the accumulation of toxic by-products. Once the culture entered stationary growth, utilization of the substrates stopped.

3.D. Utilization of unused Union Carbide UCAR deicing/anti-icing fluid

3.D.1. Growth of *P. fluorescens* on UCAR XL-54 and UCAR Ultra at 25°C

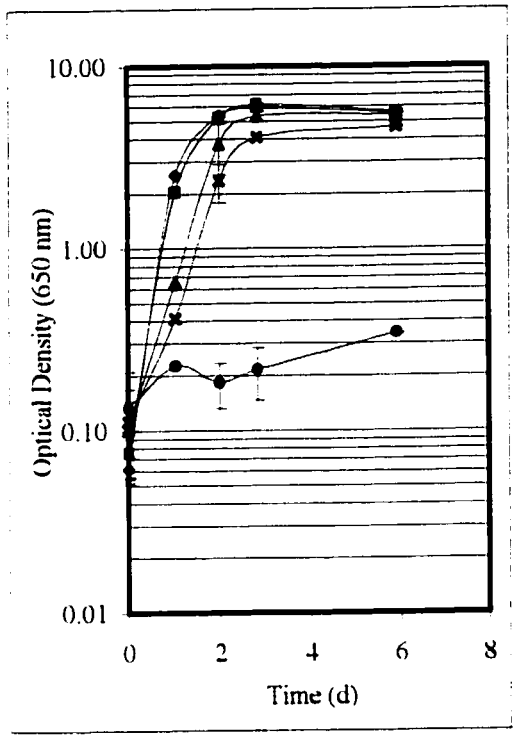
Union Carbide deicing/anti-icing fluids are ethylene glycol based formulations. The ethylene glycol concentrations for UCAR XL-54 and UCAR Ultra are 54% and 64%, respectively. In addition to ethylene glycol and water, the formulations contain additives such as corrosion inhibitors and colorants. Although these additives are present at very low concentrations, their impact upon *P. fluorescens* needed to be assessed. The growth of *P. fluorescens* with the deicing/anti-icing fluids was tested (Fig. 13). Also presented in this figure for comparison is the growth of *P. fluorescens* on pure ethylene glycol. The corresponding generation times are presented in Table 3. The data demonstrate the ability of *P. fluorescens* to grow on the deicing/anti-icing fluids over a wide range of glycol concentrations. The XL-54 formulation appeared to be more inhibitory at the higher concentrations compared to the Ultra formulation. The reason for this is unclear due to the proprietary nature of the deicing/anti-icing fluid contents. The response of the microorganism to the low and moderate concentrations of deicing/anti-icing fluids was very similar to pure ethylene glycol, thus suggesting that the additives present in the formulations did not impart a negative effect upon growth. For all future experiments, an initial glycol concentration of 10,000 mg/L was chosen.

3.D.2. Utilization of 10,000 mg/L unused UCAR deicing/anti-icing fluids at 25°C and 5°C

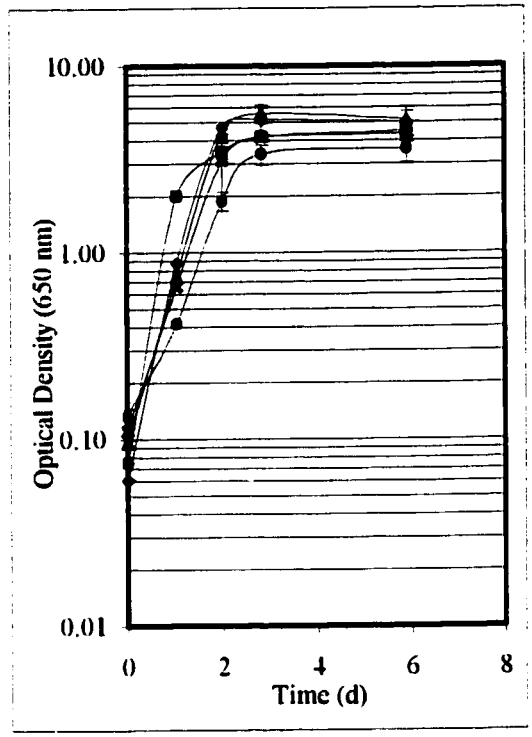
From Figure 13 and Table 3, it is evident that growth was indeed possible with UCAR XL-54 or UCAR Ultra as the sole carbon source. In order to be certain that the additives did not have any adverse effects upon *P. fluorescens*, it was also necessary to investigate the utilization of ethylene glycol from the deicing/anti-icing fluids. In

Figure 13: Growth of *P. fluorescens* on UCAR XL-54, UCAR Ultra and ethylene glycol at 25°C. Growth was monitored spectrophotometrically. For each substrate the concentrations are as follows: 10,000 mg/L (◆), 25,000 mg/L (■), 50,000 mg/L (▲), 75,000 mg/L (✖), 100,000 mg/L (○).

XL-54



Ultra



Ethylene Glycol

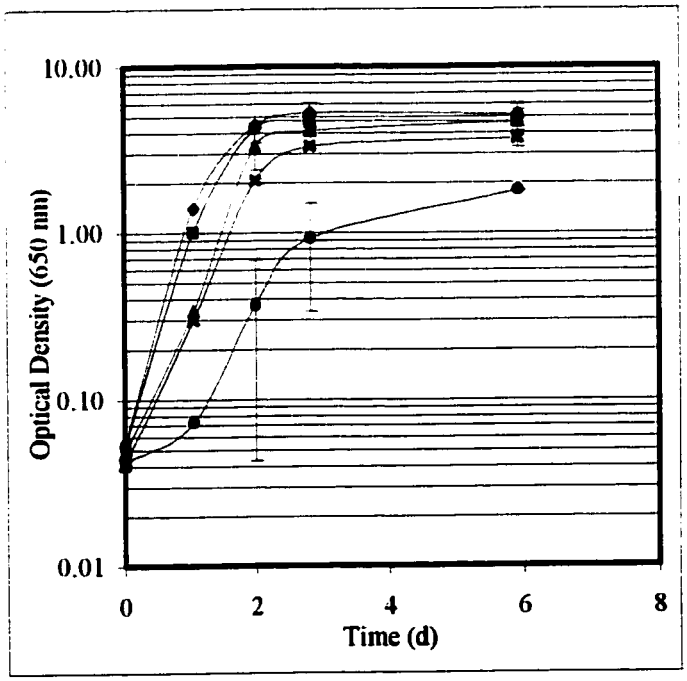


Table 3: Generation times for growth on unused UCAR deicing/anti-icing fluids and ethylene glycol.

Concentration (mg/L)	Generation Time (h)		
	XL-54	Ultra	Ethylene Glycol
10,000	6.0 ± 1.7	7.1 ± 0.9	6.4 ± 1.7
25,000	6.5 ± 1.2	7.4 ± 3.0	6.6 ± 1.3
50,000	9.4 ± 2.1	8.5 ± 0.1	8.1 ± 0.7
75,000	13.3 ± 5.9	9.8 ± 0.4	8.6 ± 0.2
100,000	73.3 ± 29.4	13.4 ± 1.4	21.3 ± 12.3

addition, due to the low dissolved oxygen levels that occur during ethylene glycol utilization, it was of interest to determine whether the addition of nitrates had any effect upon ethylene glycol utilization. The nitrates could potentially serve as an alternate electron acceptor. The cultures were set up with initial glycol concentrations of 10,000 mg/L and the experiment was conducted at both 25°C and 5°C. Inspection of the data (Fig. 14 and Table 4) suggests that the deicing fluid formulation contains some component which actually increases the utilization of the ethylene glycol (i.e. 3789 mg/L/day for XL-54 v.s. 2593 mg/L/day for pure ethylene glycol). With respect to the addition of nitrates, no effect was observed.

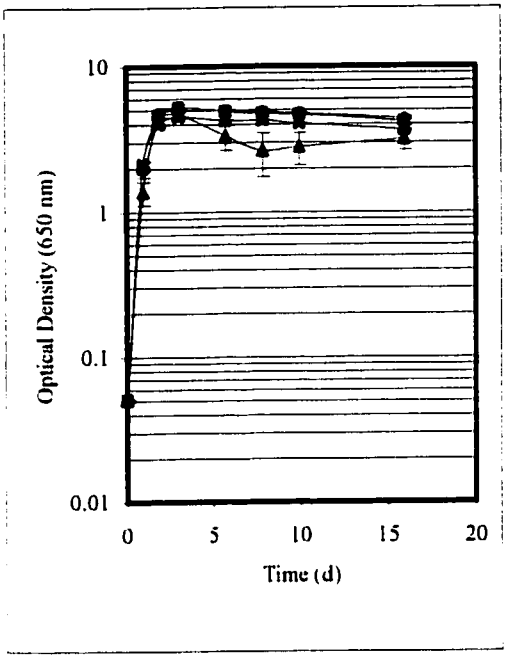
3.E. Utilization of spent UCAR deicing/anti-icing fluid

3.E.1. Growth of *P. fluorescens* on spent fluid at 25°C

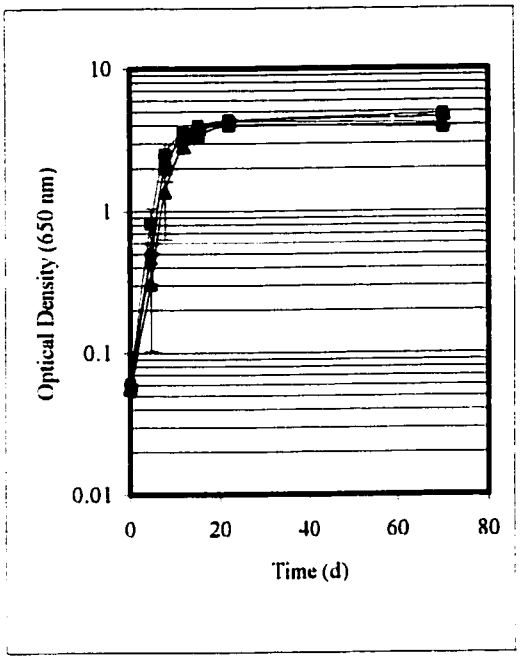
From the above experiments, the microorganism was able to utilize unused ethylene glycol-based deicing/anti-icing fluids at both 25°C and 5°C. What was yet to be determined was how the microorganism would respond to spent deicing/anti-icing fluids that likely contain a wide variety of other components such as oil and grease. To investigate this, samples of spent deicing/anti-icing fluid were taken from Pearson International Airport Terminal 3 and subjected to various biodegradation tests (Fig. 15 and Table 5). Three diluents were tested to determine the requirements for biodegradation to occur. Those diluents were BSM, Borden groundwater, and supplemented Borden groundwater. To study the utilization of ethylene glycol from the spent fluids, each spent fluid was diluted such that the initial glycol content was approximately 10,000 mg/L. Preliminary experiments indicated that the spent fluid contained microorganisms that were capable of growth when the concentration of the glycols was reduced to lower levels. Therefore, it was important to determine the contribution to overall glycol degradation that the natural population was providing. To accomplish this, three conditions were established: 1. unsterile/uninoculated (to assess

Figure 14: Growth and utilization of unused UCAR deicing/anti-icing fluids at 25°C and 5°C. The initial concentration of deicing/anti-icing fluids used was 10,000 mg/L (ethylene glycol w/v). In addition to standard BSM, BSM supplemented with 10 mM $NaNO_3$ was used. Growth was monitored spectrophotometrically and glycol removal was determined by gas chromatography. (XL-54 in standard BSM (■), XL-54 in nitrate BSM (◆), Ultra in standard BSM (✖), Ultra in nitrate BSM (▲))

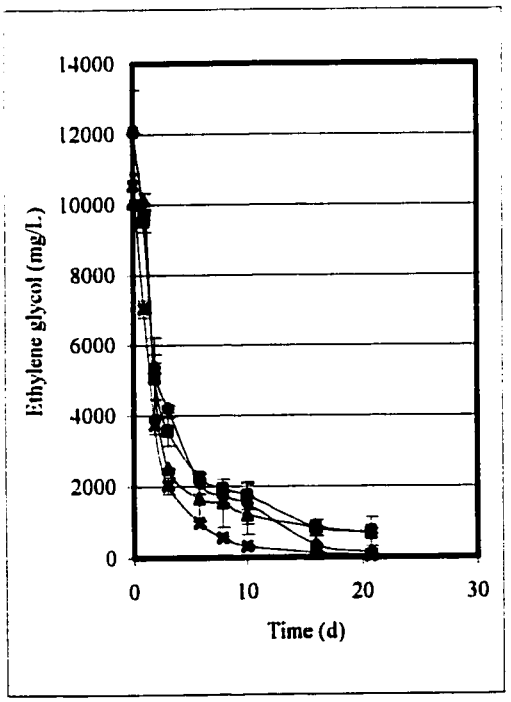
25°C


Optical Density (650 nm)
10
1
0.1
0.01
0
5
10
15
20
Time (d)

5°C


Optical Density (650 nm)
10
1
0.1
0.01
0
20
40
60
80
Time (d)

25°C


Ethylene glycol (mg/L)
14000
12000
10000
8000
6000
4000
2000
0
0
10
20
30
Time (d)

5°C

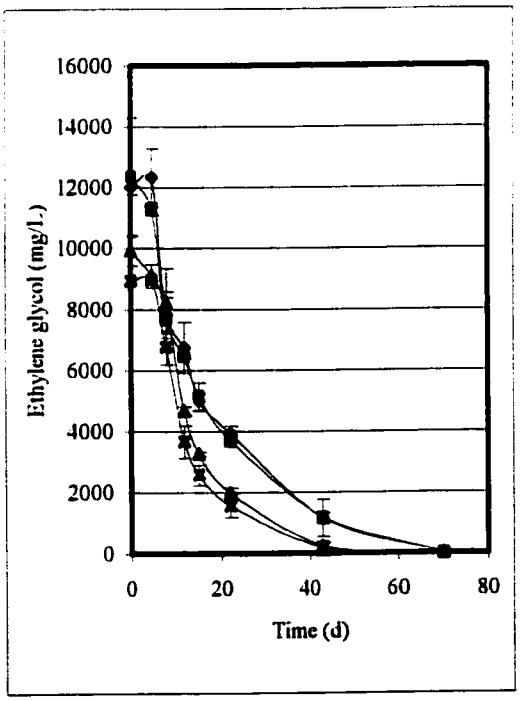
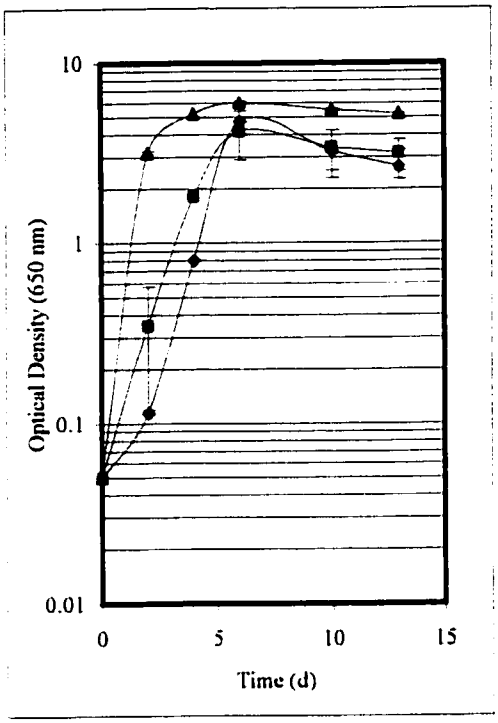

Ethylene glycol (mg/L)
16000
14000
12000
10000
8000
6000
4000
2000
0
0
20
40
60
80
Time (d)

Table 4: Growth and utilization of ethylene glycol in the presence of unused UCAR deicing/anti-icing fluids.

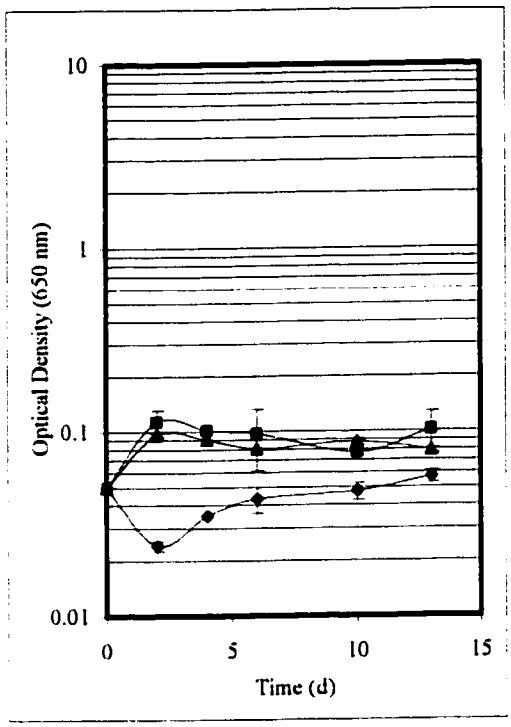
Substrate	Generation Time		Utilization (mg/L/day)	
	25°C	5°C	25°C	5°C
XL-54	6.8 ± 0.2	35.6 ± 2.0	3789 ± 1014	558 ± 54
XL-54 (Supplemented)	6.7 ± 0.1	37.7 ± 0.6	4017 ± 1432	654 ± 12
Ultra	7.0 ± 0.1	36.9 ± 2.3	3721 ± 86	638 ± 8
Ultra (Supplemented)	6.9 ± 0.1	42.9 ± 7.6	3678 ± 174	575 ± 38

Figure 15: Growth of *P. fluorescens* on spent fluid at 25°C. Initial glycol concentration was set to 10,000 mg/L. To dilute the spent fluid to the appropriate concentration, the following diluents were used: 1. basal salts media, 2. Borden groundwater, 3. supplemented Borden groundwater. To assess the activity of the natural population, the following three conditions were established: 1. natural population only (◆), 2. natural population and *P. fluorescens* (■), 3. *P. fluorescens* only (▲).

BSM



Borden groundwater



Supplemented Borden groundwater

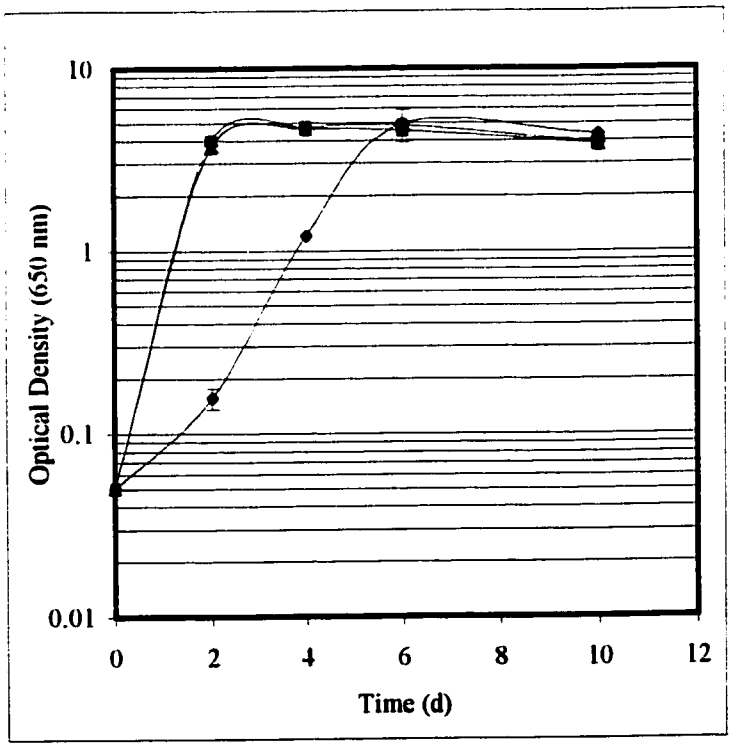


Table 5: Generation time and utilization of ethylene glycol during growth on spent UCAR deicing/anti-icing fluids.

Condition/Diluent	Generation Time (h)		Utilization (mg/L/day)	
	25°C	5°C	25°C	5°C
BSM				
Unsterile/uninoculated	17.9 ± 0.6	NG[a]	2319 ± 107	NU[b]
Unsterile/inoculated	27.9 ± 11.1	43.0 ± 4.2	1885 ± 968	394 ± 62
Sterile/inoculated	8.0 ± 0.0	40.9 ± 2.0	2522 ± 137	334 ± 19
Borden Groundwater				
Unsterile/uninoculated	NG	NG	NU	NU
Unsterile/inoculated	NG	NG	NU	NU
Sterile/inoculated	NG	NG	NU	NU
Supplemented Borden Groundwater				
Unsterile/uninoculated	19.3 ± 0.4	NG	2577 ± 49	NU
Unsterile/inoculated	7.6 ± 0.1	35.1 ± 0.7	2974 ± 94	392 ± 39
Sterile/inoculated	7.7 ± 0.1	35.1 ± 0.5	2822 ± 184	419 ± 20

[a] NG = no growth
[b] NU = no utilization

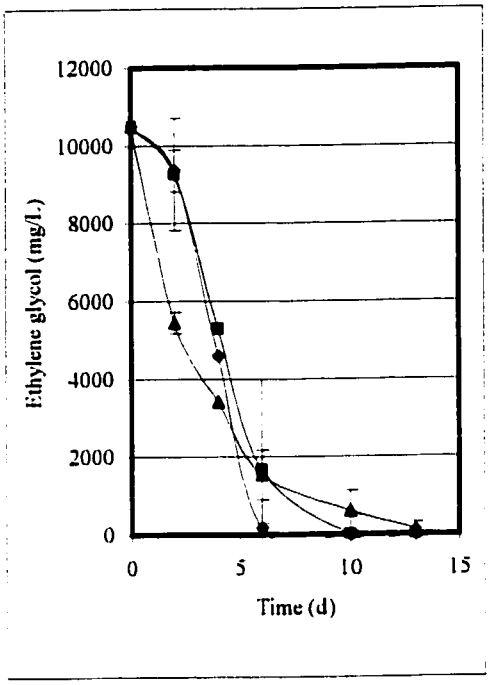
the degradative abilities of the natural population in the absence of *P. fluorescens*), 2. unsterile/inoculated (to assess whether the added *P. fluorescens* had an effect on degradation compared to the natural population alone) and 3. sterile/inoculated (to assess the degradative abilities of *P. fluorescens* without the influence of the natural population). Inorganic supplements were clearly required for growth, whether present in the BSM or in the supplemented Borden groundwater (Fig. 15). The spent fluid cannot simply be diluted with water to achieve a condition where degradation occurs. The natural population was capable of growth, however, it was slower compared to the *P. fluorescens*. Upon sterilization of the spent fluid, the added *P. fluorescens* exhibited a generation time that was very similar to growth on pure ethylene glycol in BSM at 25°C (i.e. 8.0 h in diluted spent fluid v.s. 7.5 h in ethylene glycol-BSM). A comparison of the BSM diluted cultures and supplemented Borden groundwater cultures indicated that the growth response was quite similar except for the flasks containing both the natural population and the *P. fluorescens*; the supplemented Borden groundwater yielded a shorter generation time when the natural population and *P. fluorescens* were together.

3.E.2. Utilization of spent fluid by *P. fluorescens* at 25°C

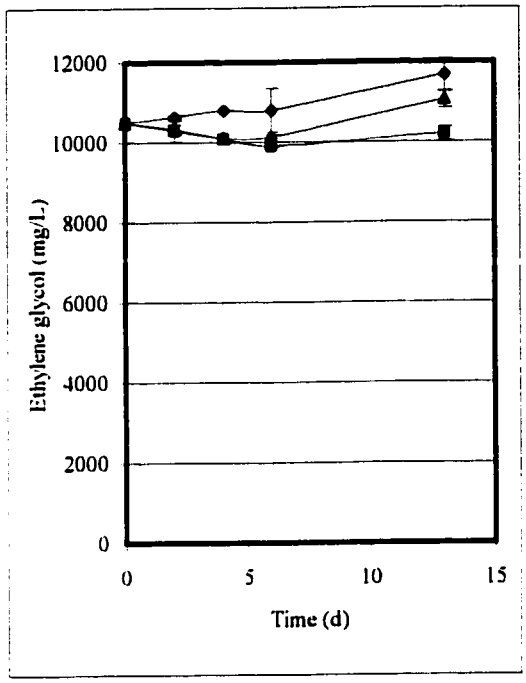
The utilization of ethylene glycol in the spent fluid experiments outlined above is presented in Figure 16 and Table 5. The results correlate quite well with the growth observed. Ethylene glycol was not utilized when the spent fluid was diluted with unsupplemented Borden groundwater. In the unsterile/uninoculated condition, the natural population was very effective with respect to ethylene glycol removal. In the BSM diluted cultures, the natural population utilized the ethylene glycol at a rate of 2319 mg/L/day; in the supplemented Borden groundwater cultures, the rate was similar at 2522 mg/L/day (Table 5). Utilization rates in the BSM and supplemented Borden groundwater cultures were similar, except when both the natural population and *P. fluorescens* were

Figure 16: Utilization of spent fluids at 25°C. Initial glycol concentration was set to 10,000 mg/L. To dilute the spent fluid to the appropriate concentration, the following diluents were used: 1. basal salts media, 2. Borden groundwater, 3. supplemented Borden groundwater. To assess the activity of the natural population, the following three conditions were established: 1. natural population only (◆), 2. natural population and *P. fluorescens* (■), 3. *P. fluorescens* only (▲).

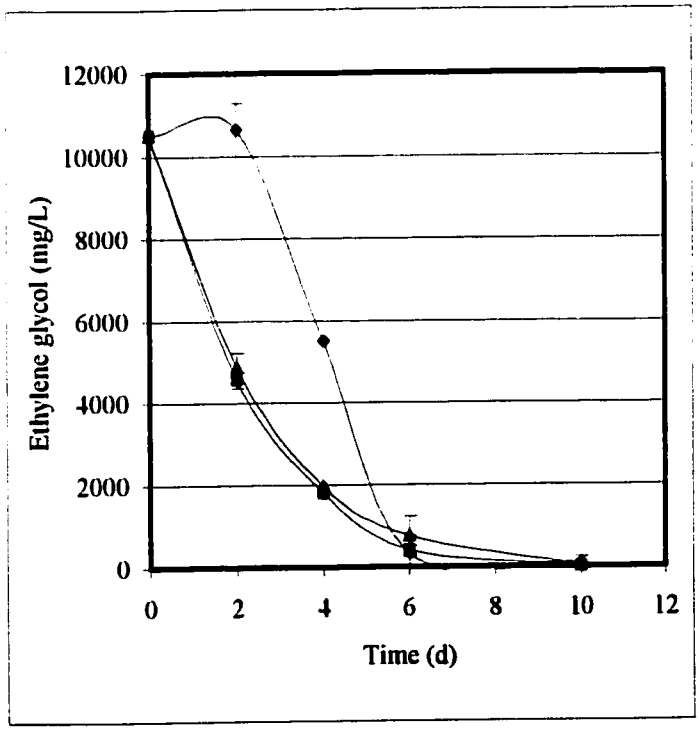
BSM



Borden groundwater



Supplemented Borden groundwater



present. As was previously observed for growth, utilization rates were enhanced in the supplemented Borden groundwater compared to the BSM when both the natural population and *P. fluorescens* were present together. Also worth noting is that the utilization rates observed were quite similar to the 2593 mg/L/day obtained with *P. fluorescens* grown under the same conditions on 10,000 mg/L pure ethylene glycol in BSM (Table 1).

3.E.3. Growth of *P. fluorescens* on spent fluid at 5°C

The growth of *P. fluorescens* on the spent deicing/anti-icing fluid was tested at 5°C (Fig. 17 and Table 5). Similar to the experiment at 25°C, growth did not occur when the spent fluid was simply diluted with water; a supplemented diluent was required. However, while the natural population present in the spent fluid grew at 25°C, at 5°C, no growth was observed in those flasks devoid of the psychrotrophic *P. fluorescens*. Furthermore, generation times in *P. fluorescens* inoculated cultures were shorter than those measured using pure ethylene glycol in BSM (i.e. 40.9 h for unsterile/inoculated vs. 56.6 h for ethylene glycol-BSM). As was also observed at 25°C, supplemented Borden groundwater favoured a shorter generation time compared to BSM at 5°C.

3.E.4. Utilization of spent fluid by *P. fluorescens* at 5°C

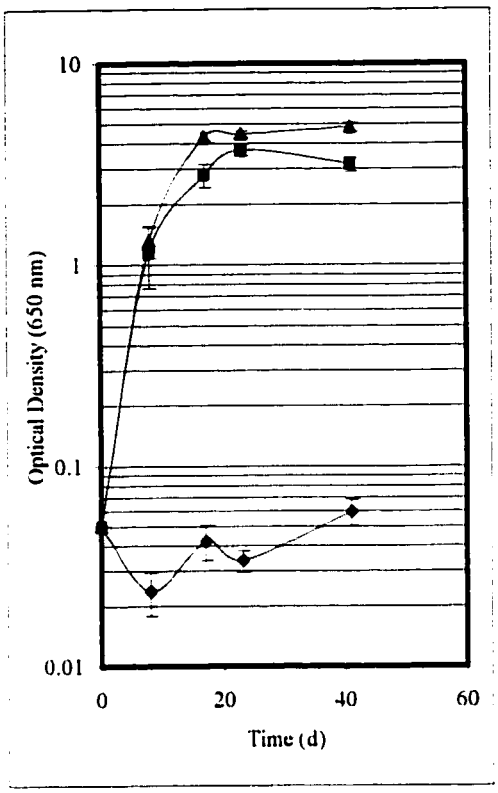
The utilization results at 5°C correlated closely with the growth noted in Section 3.E.3 (Fig. 19 and Table 5). In cases where growth was not observed, there was a corresponding absence of ethylene glycol utilization. Although the generation times for these experiments were shorter than pure ethylene glycol-BSM experiments, the rates of utilization were slightly slower. For *P. fluorescens* grown on 10,000 mg/L ethylene glycol in BSM, the rate of utilization was 422 mg/L/day whereas for the spent fluid experiments, the rates ranged from 334 mg/L/day to 419 mg/L/day.

3.E.5. Natural population in spent fluid

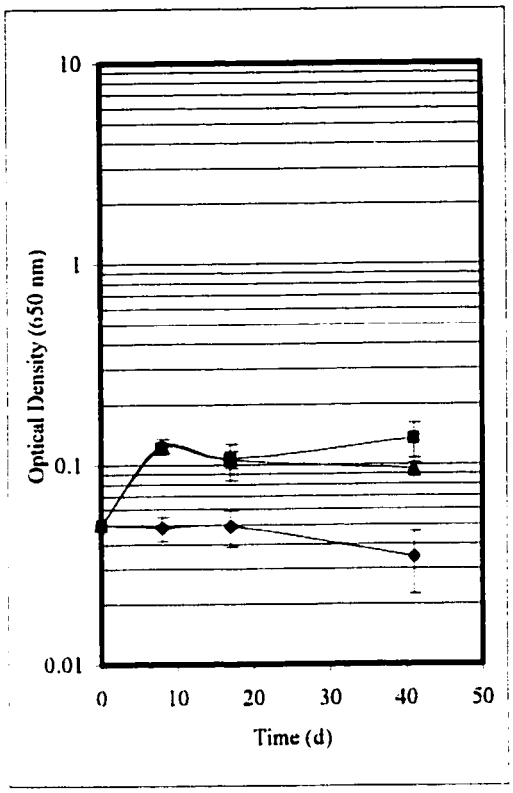
Spent fluid collected at Pearson International Airport Terminal 3 was used to isolate microorganisms capable of ethylene glycol utilization. Six isolates were purified and

Figure 17: Growth of *P. fluorescens* on spent fluid at 5°C. Initial glycol concentration was set to 10,000 mg/L. To dilute the spent fluid to the appropriate concentration, the following diluents were used: 1. basal salts media, 2. Borden groundwater, 3. supplemented Borden groundwater. To assess the activity of the natural population, the following three conditions were established: 1. natural population only (◆), 2. natural population and *P. fluorescens* (■), 3. *P. fluorescens* only (▲).

BSM



Borden groundwater



Supplemented Borden groundwater

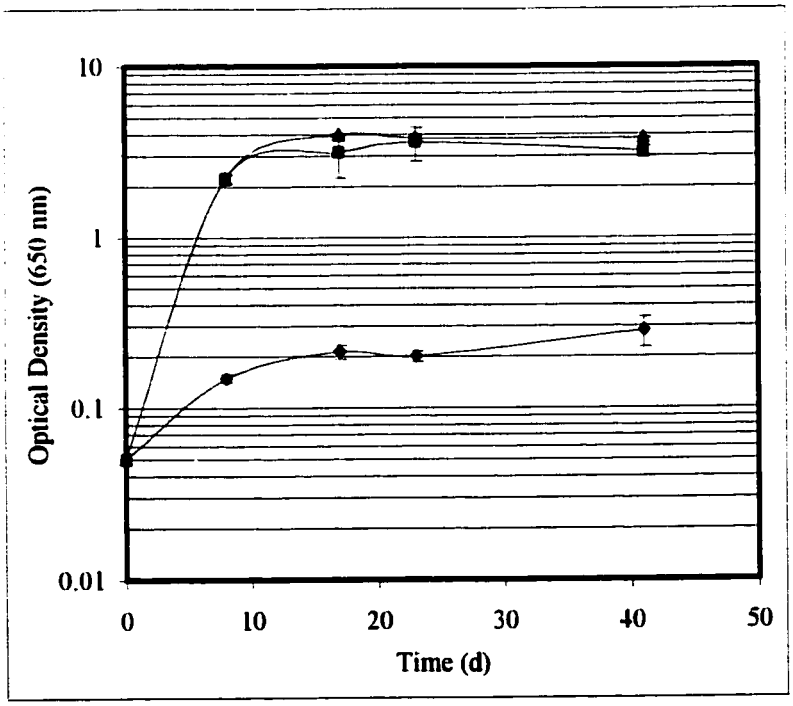
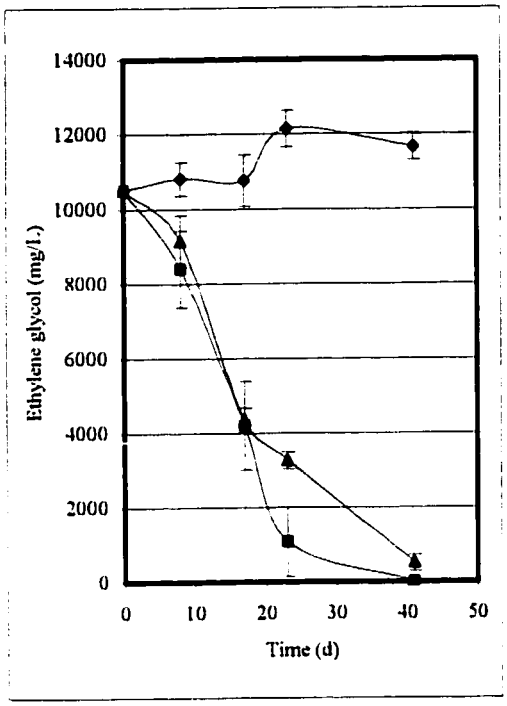
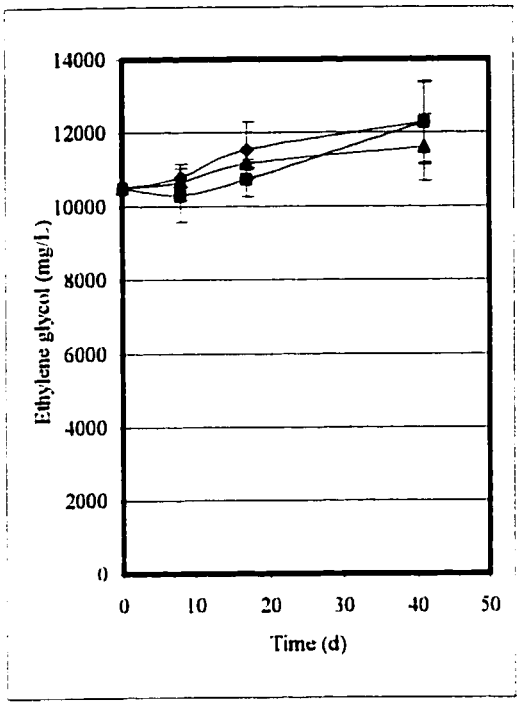


Figure 18: Utilization of spent fluids at 5°C. Initial glycol concentration was set to 10,000 mg/L. To dilute the spent fluid to the appropriate concentration, the following diluents were used: 1. basal salts media, 2. Borden groundwater, 3. supplemented Borden groundwater. To assess the activity of the natural population, the following three conditions were established: 1. natural population only (◆), 2. natural population and *P. fluorescens* (■), 3. *P. fluorescens* only (▲).

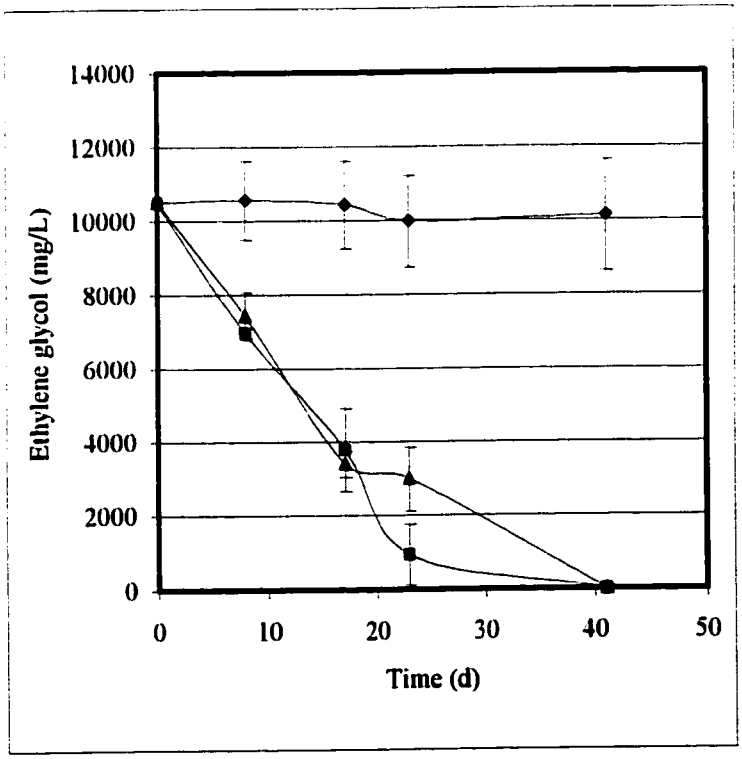
BSM



Borden groundwater



Supplemented Borden groundwater



analyzed using the BioLog Microstation System. From the analysis, it was determined that four belonged to *P. fluorescens* Type B and one culture belonged to *P. fluoresc*ens Type G. The remaining culture could not be identified using the Biolog system.

3.F. Oxygen consumption – Determination of oxidase induction

3.F.1. Oxidase activity in response to ethylene glycol and propylene glycol

The alcohol oxidase activity that mediates the initial metabolic steps in the utilization of ethylene glycol and propylene glycol appears to result from two separate enzymes that are specific for each substrate. From the data presented in Table 6, it is evident that growth on propylene glycol does not induce an oxidase capable of ethylene glycol oxidation.

3.G. Uptake studies involving ^{14}C-ethylene glycol

3.G.1. Effect of culture age upon ^{14}C-ethylene glycol transport

To conduct ^{14}C-ethylene glycol uptake studies, it was necessary to determine whether variations in culture age would affect the rates of transport observed (Fig. 19 and Table 7). It was evident that ^{14}C-ethylene glycol uptake was affected by the age of the culture. Maximal uptake was observed during the early exponential phase of growth (OD_{650} = 0.284). As the culture entered the mid-exponential phase of growth, the relative uptake decreased to 53%. The rate of ^{14}C-ethylene glycol uptake was then relatively constant through to late-exponential, after which the decline was more significant. In all experiments where culture age was not a parameter being studied, every effort was taken to sample at OD_{650} = 0.8 to 1.2.

3.G.2. Induction response of the ethylene glycol transport mechanism

When *P. fluorescens*, grown on glucose or glycolate, is transferred to a medium containing ethylene glycol as the sole carbon source, there is a lag period associated with the acclimation to the new substrate. To determine whether the ethylene glycol transport mechanism is specific and requires induction, cells were grown on glucose and glycolate and tested for ^{14}C-ethylene glycol uptake (Fig. 20). With cells that were grown on

Table 6: Oxygen consumption for selected substrates for cells grown on ethylene glycol and propylene glycol.

Growth Substrate	Test Condition	O_2 consumption ($\mu l\ O_2\ hr^{-1}$)
ethylene glycol	endogenous respiration	3.0 ± 1.5
	1% ethylene glycol	41.0 ± 1.8
propylene glycol	endogenous respiration	16.2 ± 6.5
	1% propylene glycol	39.8 ± 6.1
	1% ethylene glycol	12.4 ± 1.7

Figure 19: The effect of culture age on ethylene glycol uptake. Uptake was measured using ^{14}C-ethylene glycol. (early exponential (◆), mid exponential (■), late exponential I (▲), late exponential II (✱), decline (○))

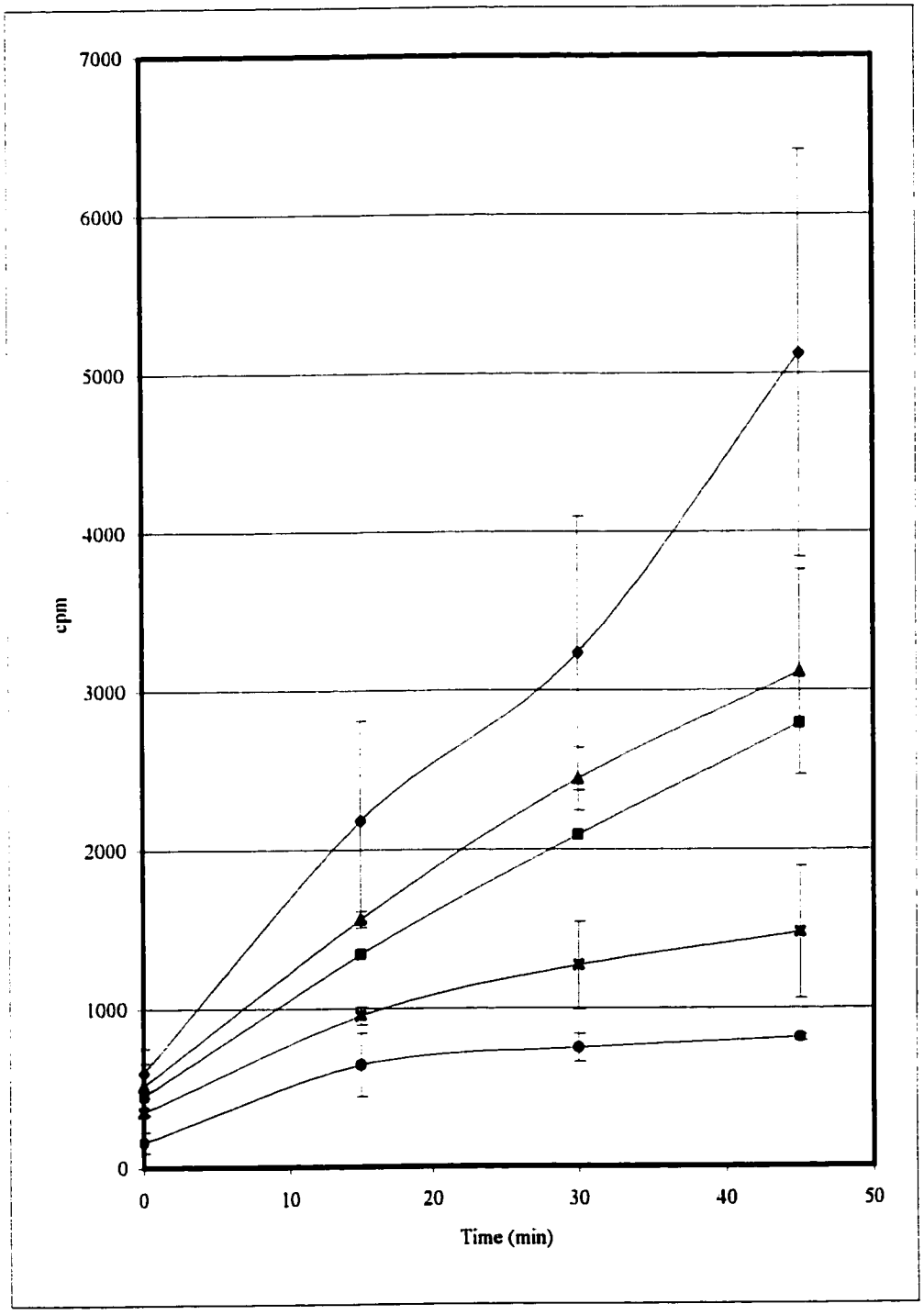
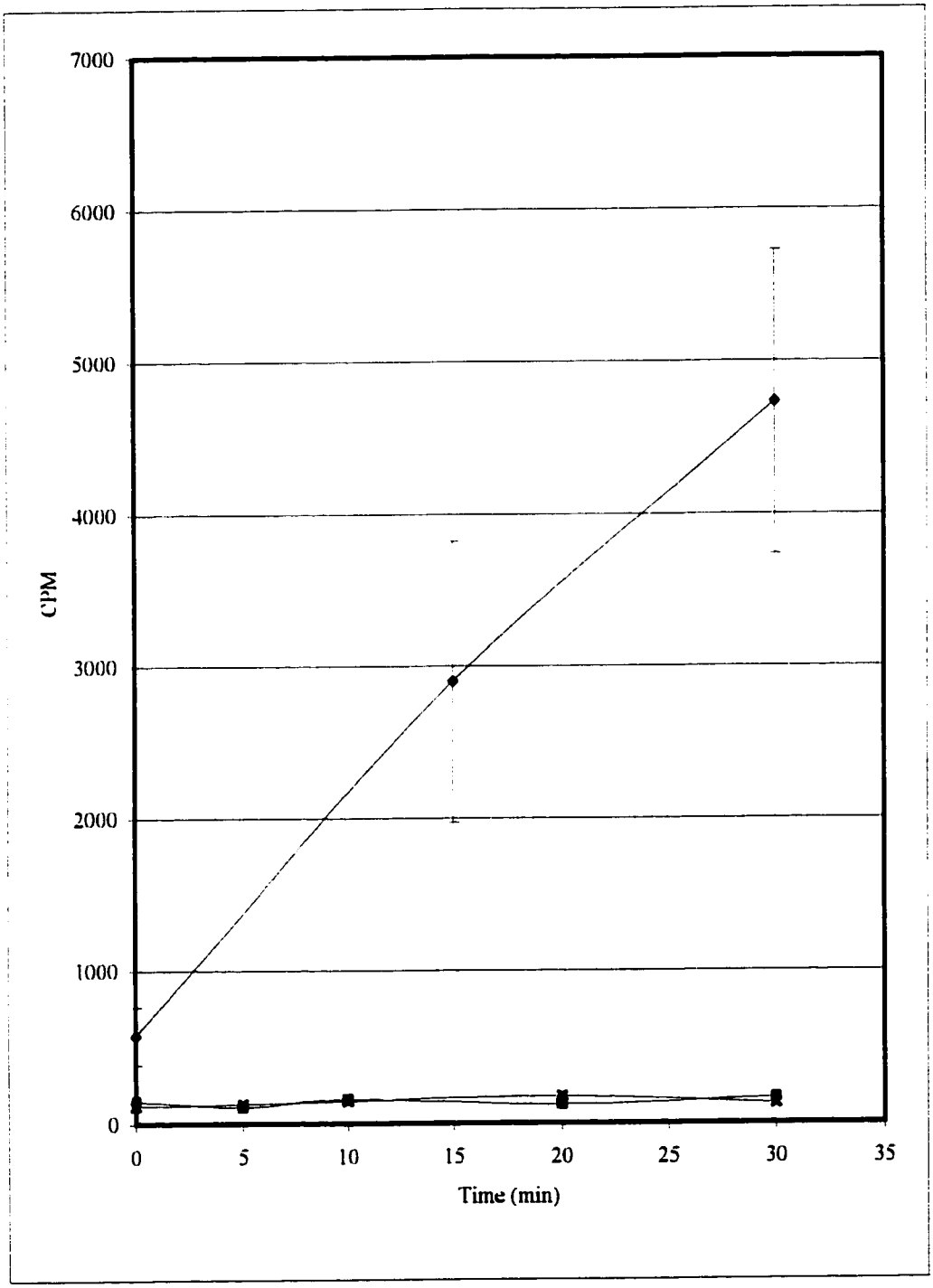

7000
6000
5000
4000
3000
2000
1000
0
cpm
0
10
20
30
40
50
Time (min)

Table 7: Effect of culture age upon ^{14}C-ethylene glycol uptake.

Culture Age	Uptake (cpm/min)	Relative Uptake (%)
Early-exp. (O.D. = 0.284)	98 ± 24	100 ± 24
mid-exp. (O.D. = 2.8)	52	53
late-exp. (I) (O.D. = 5.3)	65 ± 2	66 ± 2
late-exp. (II) (O.D. = 5.7)	31 ± 9	32 ± 9
decline (O.D. = 5.0)	20 ± 0.7	20 ± 0.7

Figure 20: The induction response of the ethylene glycol transport mechanism. Uptake was measured using ^{14}C-ethylene glycol. (glycolate grown cells (■), glucose grown cells (✖), ethylene glycol grown cells (◆))


7000
6000
5000
4000
3000
2000
1000
0
CPM
0
5
10
15
20
25
30
35
Time (min)

ethylene glycol, maximal uptake was recorded. However, when *P. fluorescens* was grown on glucose or glycolate, the relative rates of ^{14}C-ethylene glycol uptake decreased to 0.4% and 0.5%, respectively. Thus, it appears that ethylene glycol transport is an inducible system.

3.G.3. Effect of transport inhibitors upon ^{14}C-ethylene glycol transport

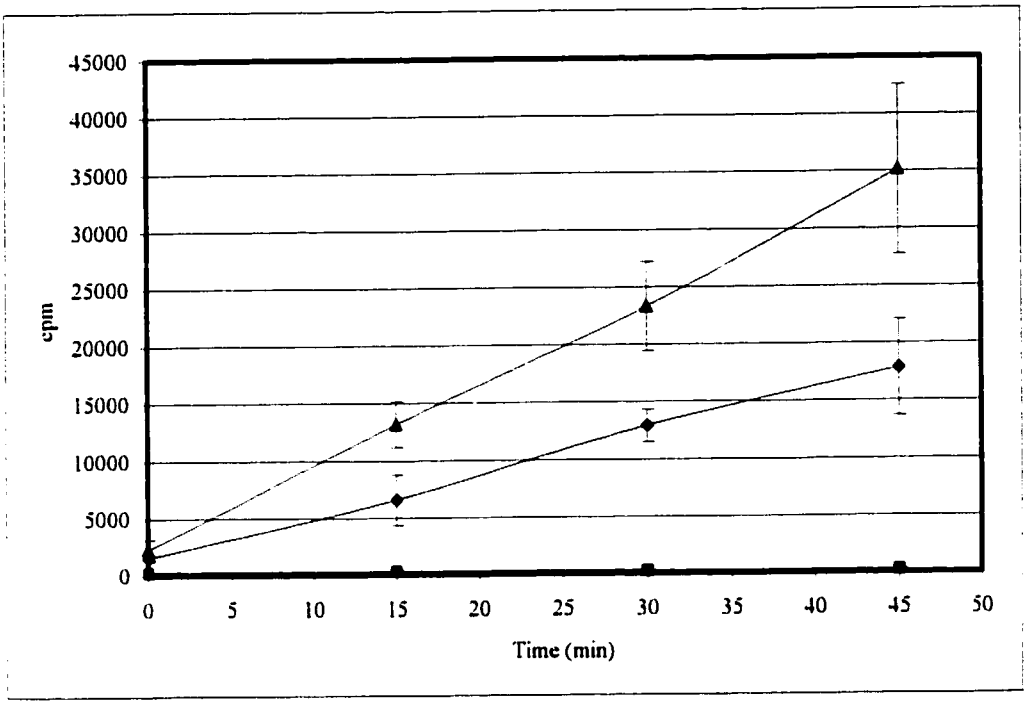
The apparent inducible character of ethylene glycol uptake suggests that the process involves active transport. To test this, two transport inhibitors were utilized. The first, 2,4-dinitrophenol (DNP), is an uncoupler and inhibitor of proton-motive force. The second, N,N'-dicyclohexylcarbodiimide (DCCD), is an inhibitor of membrane-bound ATPase. The presence of either DNP or DCCD clearly led to a decrease in the uptake of ^{14}C-ethylene glycol (Fig. 21 and Table 8). For both inhibitors, a concentration of 0.1mM inhibited transport by approximately 50%. When the concentration of DNP and DCCD are increased to 1.0 mM, the inhibition of transport increases to 100% and 88% respectively. Therefore, the transport process by which ethylene glycol enters the cell is energy dependent, suggesting the presence of active transport.

3.G.4. Effect of pH upon ^{14}C-ethylene glycol uptake

To better understand the dynamics of ethylene glycol uptake, factors that could potentially affect the uptake mechanism were investigated. The first factor to be tested was pH (Fig. 22 and Table 9). Previous growth experiments had demonstrated that optimal growth of *P. fluorescens* occurred at pH 7 (data not shown). Consequently, it was expected that an optimal uptake of the target substrate would occur at a similar pH. Analysis of the uptake data indicate that uptake was indeed maximal at pH 7. At an alkaline pH (pH 8), the relative uptake decreased to 17%. Conversely, when the pH was more acidic, there was more tolerance. At pH 6, the relative uptake was 56% but this rate quickly decreased to 7% at pH 5. Below this pH, essentially no uptake occurred. The tolerance over the pH range of 6 – 7 was expected since it was observed

Figure 21: The effect of metabolic inhibitors on ethylene glycol uptake. Uptake was measured using ^{14}C-ethylene glycol. (Concentrations correspond to: 0.1 mM (◆), 1.0 mM (■), no inhibitor (▲))

2,4-Dinitrophenol



N,N'dicyclohexylcarbodiimide

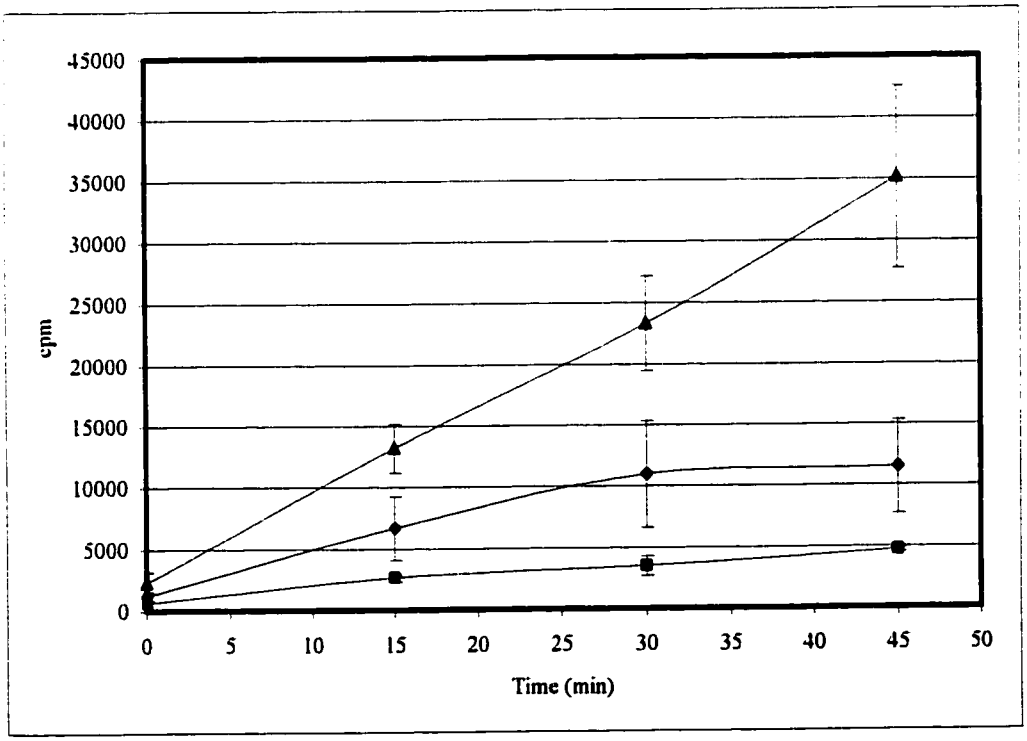


Table 8: Effect of transport inhibitors upon ^{14}C-ethylene glycol uptake.

Inhibitor	Uptake (cpm/min)	Relative Uptake (%)
Control (no inhibitor)	724 ± 148	100 ± 20
2,4-dinitrophenol		
0.1 mM	369 ± 83	51 ± 11
1.0 mM	no uptake	no uptake
N,N'dicyclohexylcarbodiimide		
0.1 mM	326 ± 133	45 ± 18
1.0 mM	87 ± 2	12 ± 0.3

Figure 22: The effects of pH upon ethylene glycol uptake. Uptake was observed using ^{14}C-ethylene glycol. (pH 4 (○), pH 5 (✖), pH 6 (▲), pH 7 (■), pH 8 (◆))

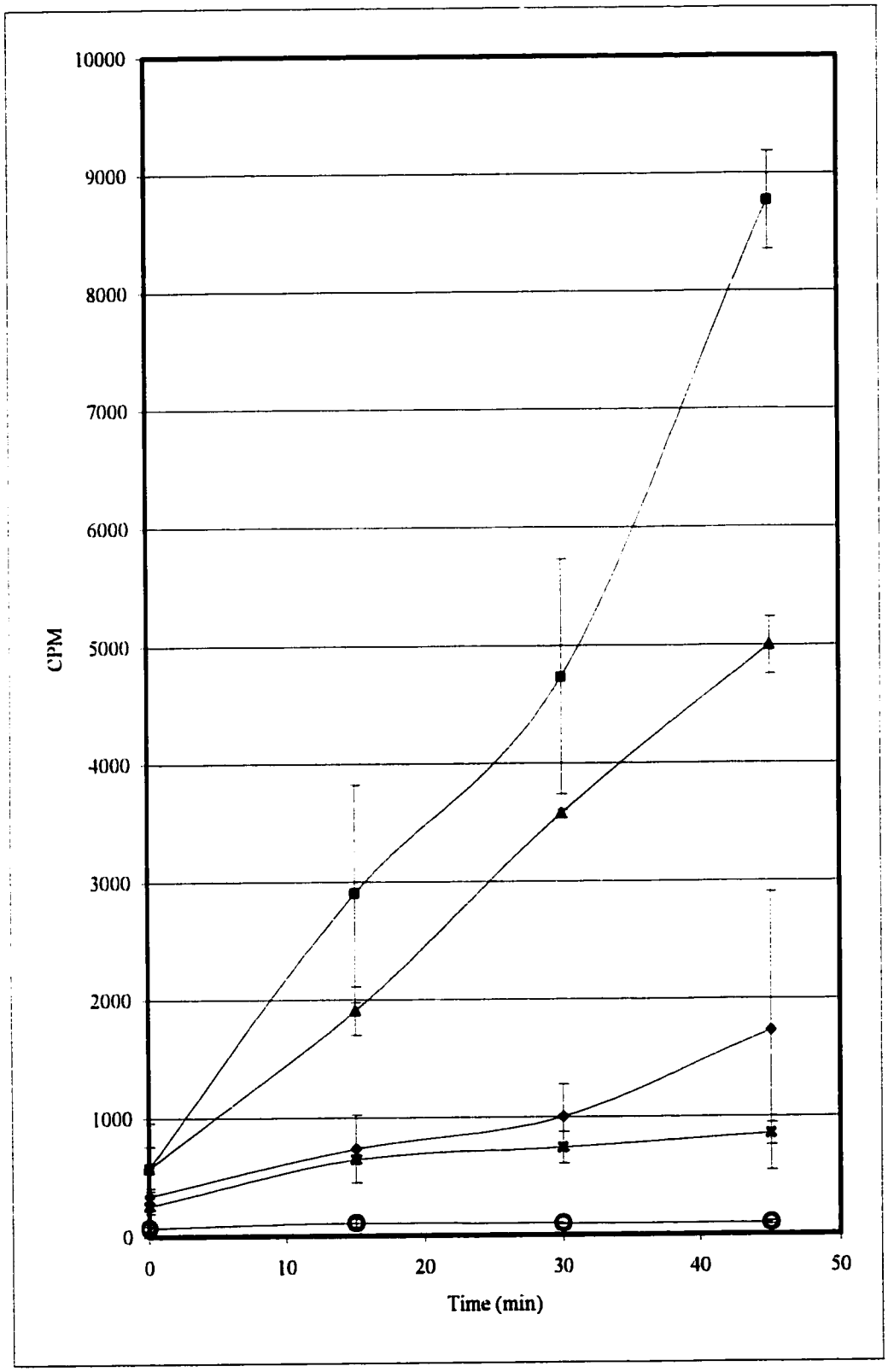

10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
CPM
0
10
20
30
40
50
Time (min)

Table 9: Effect of pH upon ^{14}C-ethylene glycol uptake.

pH	Uptake (cpm/min)	Relative Uptake (%)
7 (control)	176 ± 5	100 ± 3
4	0.5 ± 0.9	0.3 ± 0.5
5	13 ± 3	7 ± 2
6	99 ± 11	56 ± 6
8	30 ± 24	17 ± 14

that during ethylene glycol metabolism, the pH of the batch culture can fall to a pH of 5.9.

3.G.5. Effect of temperature upon ^{14}C-ethylene glycol uptake

A similar set of experiments was conducted to determine the effect of temperature upon ethylene glycol uptake. Temperatures ranging from 0°C to 35°C were investigated (Fig. 23 and Table 10). Previous experiments indicated that optimal growth occurred around 25°C. The uptake data correlated with this finding, as optimal uptake was found at 25°C. Relatively high rates of uptake were observed at temperatures ranging from 10°C to 35°C, but the relative rates fall to 52% and 26%, respectively at 5°C and 0°C.

To verify that the uptake observed was not due to nonspecific adsorption of ^{14}C-ethylene glycol to the cells, cells were boiled for 5 min to effectively eliminate any metabolic and/or transport mechanisms involved in transport processes. Boiled cells sorbed negligible radioactivity indicating the absence of nonspecific binding of the ^{14}C-ethylene glycol (Fig. 23).

3.G.6. Effect of monohydric alcohols upon ^{14}C-ethylene glycol transport

Ethylene glycol is a dihydric alcohol that is described by the general chemical formula $C_nH_{2n}(OH)_2$. Dihydric alcohols exhibit a similar chemical behaviour compared to monohydric alcohols (i.e. ethanol, butanol). Due to similarities between the two classes of alcohols, it was of interest to determine whether monohydric alcohols had any effect upon ethylene glycol transport and induction. Before conducting uptake studies, it was necessary to determine whether growth was possible on monohydric alcohols. The results of the growth experiments (Fig. 24 and Table 11) illustrate that the primary alcohols tested (ethanol, 1-butanol, 1-propanol) supported growth while secondary alcohols (i.e. 2-butanol, 2-propanol) did not. Growth did not occur in the presence of methanol.

To assess whether monohydric alcohols affected ethylene glycol transport, two experimental procedures were utilized. The first procedure involved growing the cells

Figure 23: The effects of temperature upon ethylene glycol uptake. Uptake is measured using ^{14}C-ethylene glycol. (0°C (◆), 5°C (○), 10°C (✱), 25°C (▲), 35°C (■), boiled cells (●))

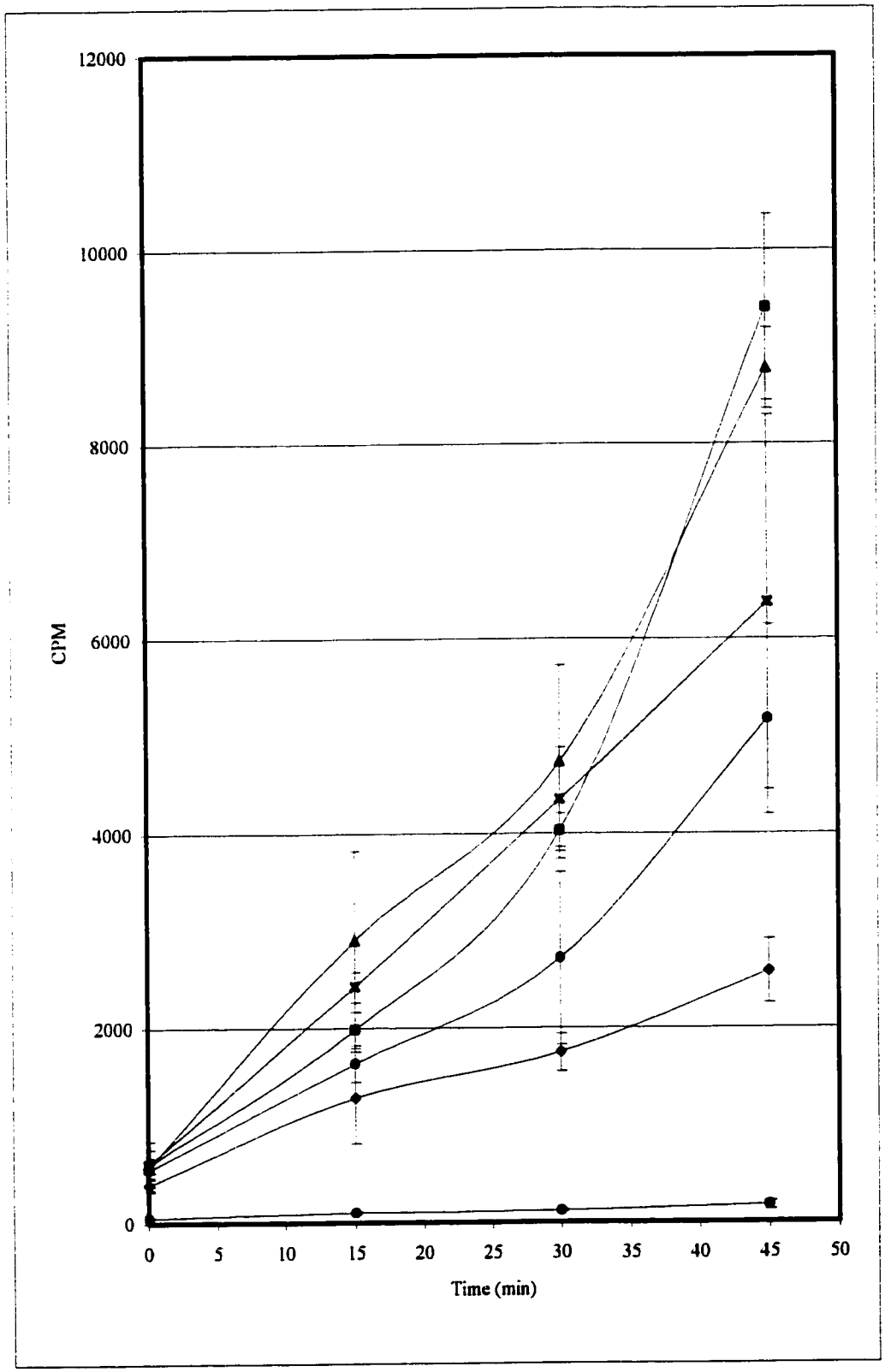

12000
10000
8000
6000
4000
2000
0
CPM
0
5
10
15
20
25
30
35
40
45
50
Time (min)

Table 10: Effect of temperature upon ^{14}C-ethylene glycol uptake.

Temperature (°C)	Uptake (cpm/min)	Relative Uptake (%)
25 (control)	176 ± 5	100 ± 3
35	114 ± 14	65 ± 8
10	118 ± 30	67 ± 17
5	92 ± 9	52 ± 5
0	45 ± 4	26 ± 2
killed cells	3 ± 0.6	2 ± 0.3

Figure 24: Growth of *P. fluorescens* on growth-supporting monohydric alcohols. Alcohols were added in equimolar (carbon) concentrations. Growth was monitored by spectrophotometry. (ethanol (◆), 1-propanol (■), 1-butanol (▲))

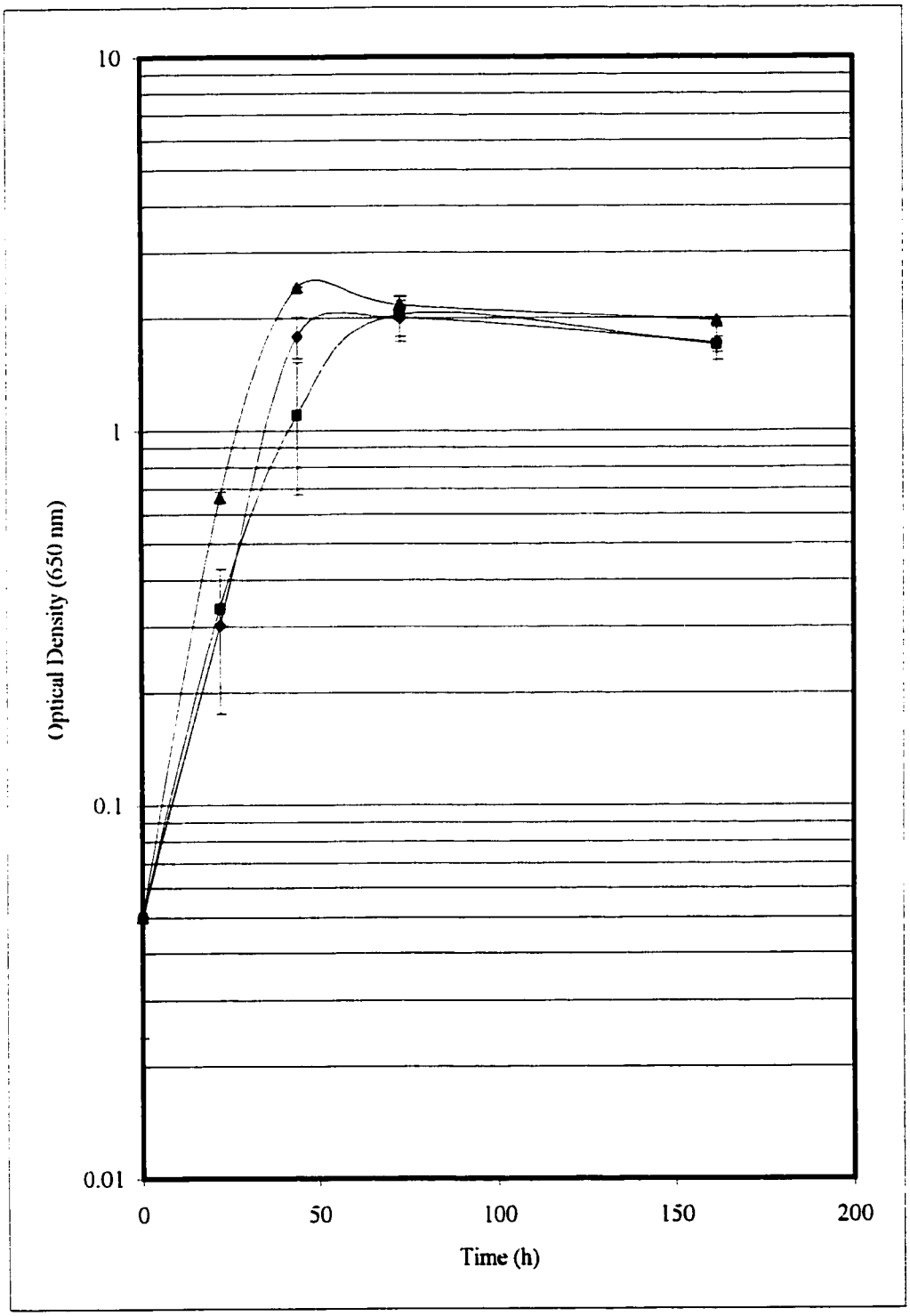

10
1
0.1
0.01
Optical Density (650 nm)
0
50
100
150
200
Time (h)

Table 11: Generation times for cells grown on selected monohydric alcohols at 25°C.

Growth Substrate	Generation Time (h)
methanol	no growth
ethanol	8.5 ± 0.3
1-propanol	10.1 ± 1.3
2-propanol	no growth
1-butanol	7.9 ± 0.0
2-butanol	no growth

on the alcohols being tested followed by the ^{14}C-ethylene glycol uptake assay. The alcohols tested were ethanol, 1-propanol and 1-butanol (Fig. 25 and Table 12). As the alcohol chain length increased, the ability to induce the ethylene glycol transport mechanism decreased. Of the alcohols tested, ethanol most closely resembles ethylene glycol and it was observed that ethanol induced the highest levels of transport with a relative uptake rate of 22%, compared to uptake by ethylene glycol-grown cells. The relative uptake rates for 1-propanol and 1-butanol were 11% and 2%, respectively. As the compound structure deviates from the basic structure of ethylene glycol, the compound decreased in its ability to induce the ethylene glycol transport mechanism.

The second experimental procedure utilized substrate competition to identify structural elements recognized by the ethylene glycol transport mechanism, regardless of whether the compound supports growth. Cells grown on ethylene glycol were tested for ^{14}C-ethylene glycol uptake while in the presence of a competitor. A significant reduction in ^{14}C-ethylene glycol uptake would indicate a strong competitor while only slight reductions indicate a weak competitor. Both primary (ethanol, 1-propanol, 1-butanol) and secondary alcohols (2-propanol, 2-butanol) were tested (Fig. 26 and Table 13). It is evident that all the alcohols effectively competed with ethylene glycol for the transport mechanism. It is interesting to note that although secondary alcohols were shown not to support growth, they did compete for the transport mechanism.

3.G.7. Effect of dihydric alcohols (diols) upon ^{14}C-ethylene glycol transport

It was of interest to determine whether certain structures of dihydric alcohols were responsible for the induction of ethylene glycol transport. As the carbon chain length of dihydric alcohols is increased, an increasing number of isomers is possible. The following diols were chosen: 1,2-ethanediol (ethylene glycol), 1,2-propanediol (propylene glycol), 1,3-propanediol, 1,2-butanediol, 1,3-butanediol, 1,4-butanediol, 2,3-butanediol, 1,2-pentanediol, 1,5-pentanediol, 2,4-pentanediol, 1,2-hexanediol. When the

Figure 25: Ethylene glycol uptake with *P. fluorescens* grown on monohydric alcohols. Uptake was measured using ^{14}C-ethylene glycol. (ethanol (◆), 1-propanol (■), 1-butanol (▲), control (✖))

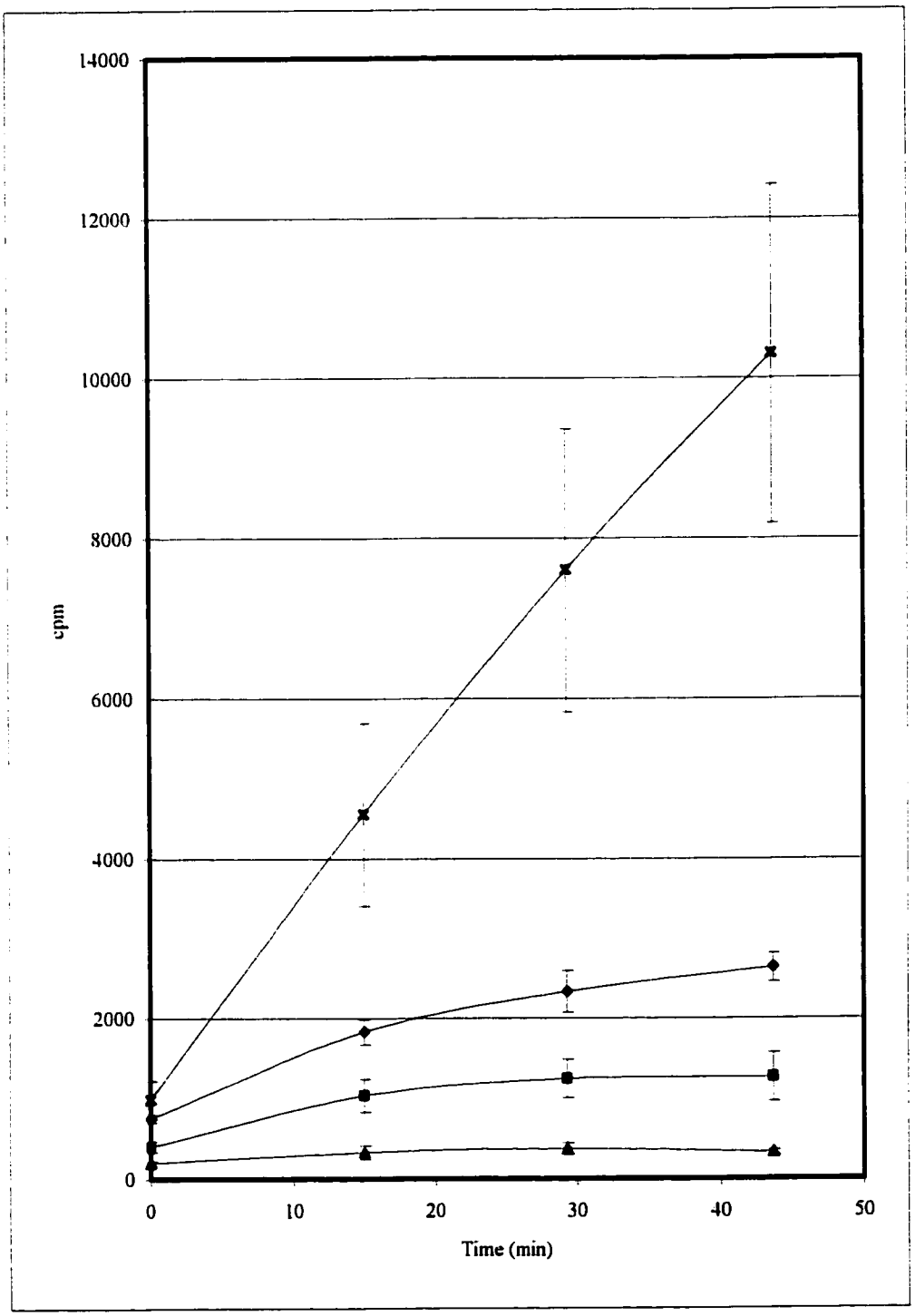
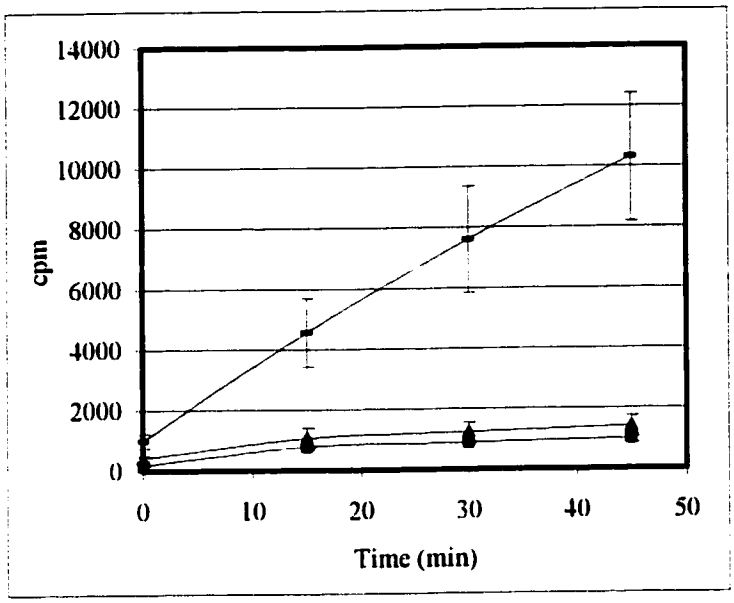

14000
12000
10000
8000
6000
4000
2000
0
cpm
0
10
20
30
40
50
Time (min)

Table 12: Induction of ^{14}C-ethylene glycol uptake by growth on selected monohydric alcohols.

Growth Substrate	Uptake (cpm/min)	Relative Uptake (%)
Ethylene glycol (control)	244 ± 32	100 ± 13
Ethanol	53 ± 7	22 ± 3
1-Propanol	28 ± 6	11 ± 2
1-Butanol	6 ± 1	2 ± 0.4

Figure 26: Competitive uptake with monohydric alcohols. Uptake was measured using ^{14}C-ethylene glycol. (no competitor added (–), ethylene glycol added (▲); Graph A: ethanol (■); Graph B: 1-propanol (■), 2-propanol (●); Graph C: 1-butanol (■), 2-butanol (●))

A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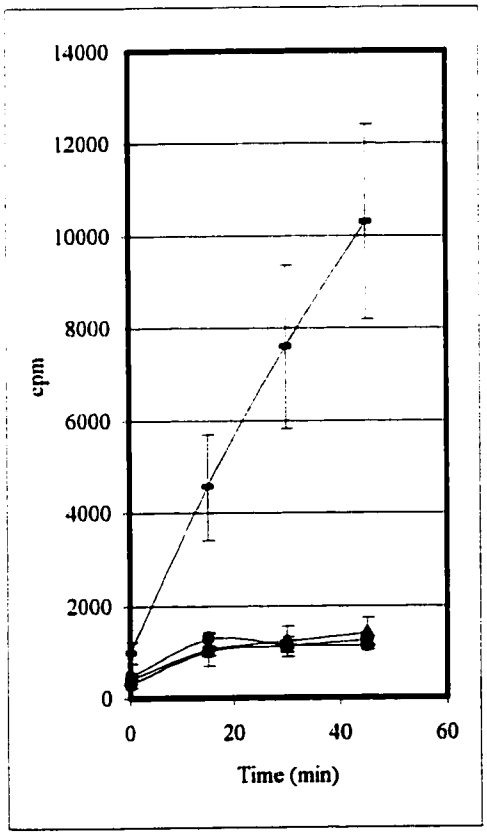
8000
6000
4000
2000
0
cpm
0
10
20
30
40
50
Time (min)

B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12000
10000
8000
6000
4000
2000
0
cpm
0
20
40
60
Time (min)

C

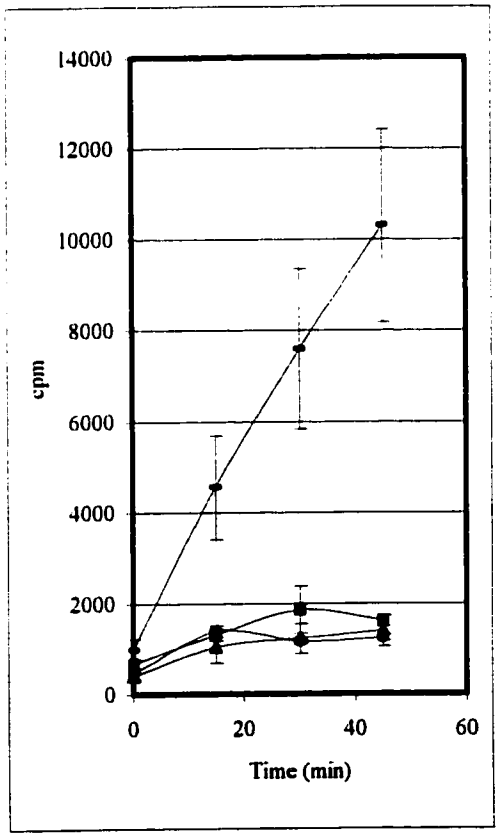

14000
12000
10000
8000
6000
4000
2000
0
cpm
0
20
40
60
Time (min)

Table 13: Competition of monohydric alcohols with ethylene glycol during ^{14}C-ethylene glycol uptake.

Competitor	Uptake (cpm/min)	Relative Uptake (%)
no competition (control)	206 ± 45	100 ± 22
Ethylene glycol	27 ± 8	13 ± 4
Ethanol	40 ± 2	19 ± 1
1-Propanol	46 ± 1	22 ± 0.5
2-Propanol	54 ± 12	26 ± 6
1-Butanol	43 ± 2	21 ± 1
2-Butanol	63 ± 15	31 ± 7

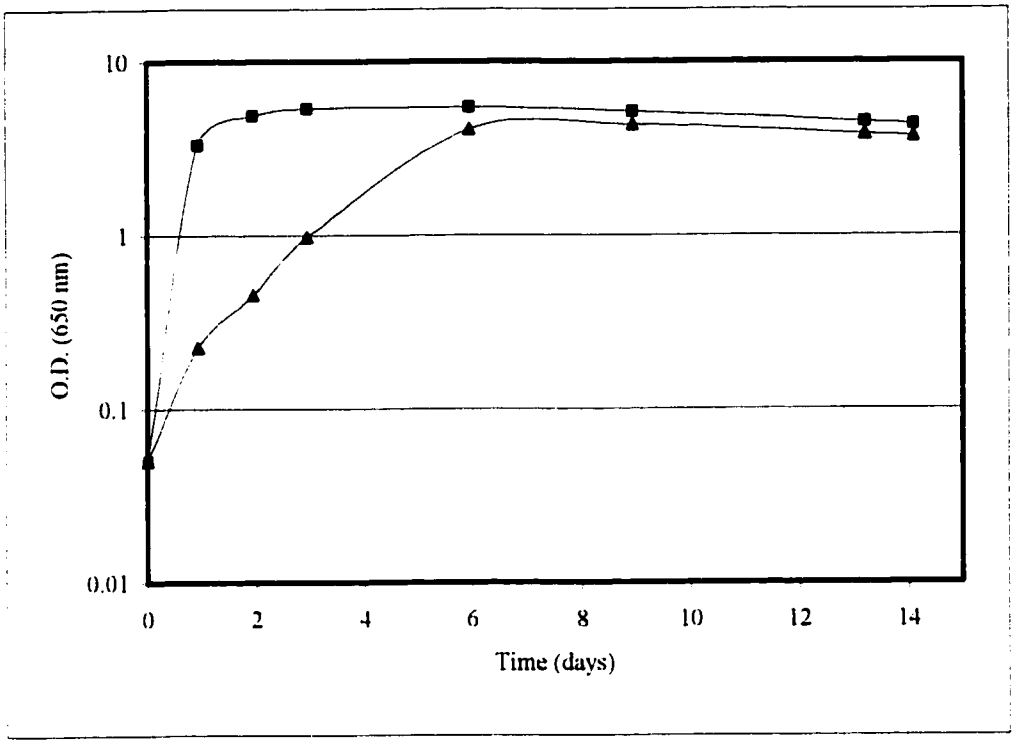
compounds were tested as potential growth substrates, it was evident that growth was limited to dihydric alcohols with carbon chain lengths of 4 or less (Fig. 27 and Table 14). Furthermore, it appeared necessary for a hydroxyl group to be located at a primary position within the molecule. This was evident within the butanediol group where those structures with a hydroxyl moiety in the primary position permitted growth whereas 2,3-butanediol did not (Fig. 27B).

To determine whether certain structural features are required to induce the ethylene glycol transport mechanism, the following dihydric alcohols were used as growth substrates and tested for ethylene glycol transport induction: 1,2-ethanediol (ethylene glycol), 1,2-propanediol, 1,3-propanediol, 1,3-butanediol and 1,4-butanediol. The data indicated that the carbon chain length influenced the induction of the uptake mechanism (Figure 28 and Table 15). As for the primary alcohols, an increase in the carbon chain length resulted in a decrease in the uptake. Furthermore, the isomers within a particular group (e.g. propanediol) result in similar levels of transport induction.

The second experimental design in which dihydric alcohols were used as competitors against ethylene glycol in ethylene glycol acclimated cells is presented in Figure 29 and Table 16. For a dihydric alcohol to be an effective competitor, the structure must exhibit a hydroxyl moiety in a primary position. With 2,3-butanediol and 2,4-pentanediol, the extent of competition was somewhat diminished compared to those dihydric alcohols containing hydroxyl groups in the primary position. Also interesting was that although isomers belonging to the pentanediol and hexanediol groups did not support growth, they were able to compete with ethylene glycol for the transport mechanism.

Figure 27: Growth of *P. fluorescens* on dihydric alcohols. Alcohols were added in equimolar (carbon) concentrations. Growth was monitored by spectrophotometry. (The data points plotted represent single values) (A: 1,2-propanediol (■), 1,3-propanediol (▲) ; B: 1,2-butanediol (◆), 1,3-butanediol (■), 1,4-butanediol (▲), 2,3-butanediol (✖)).

A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0.1
0.01
O.D. (650 nm)
0
2
4
6
8
10
12
14
Time (days)

B

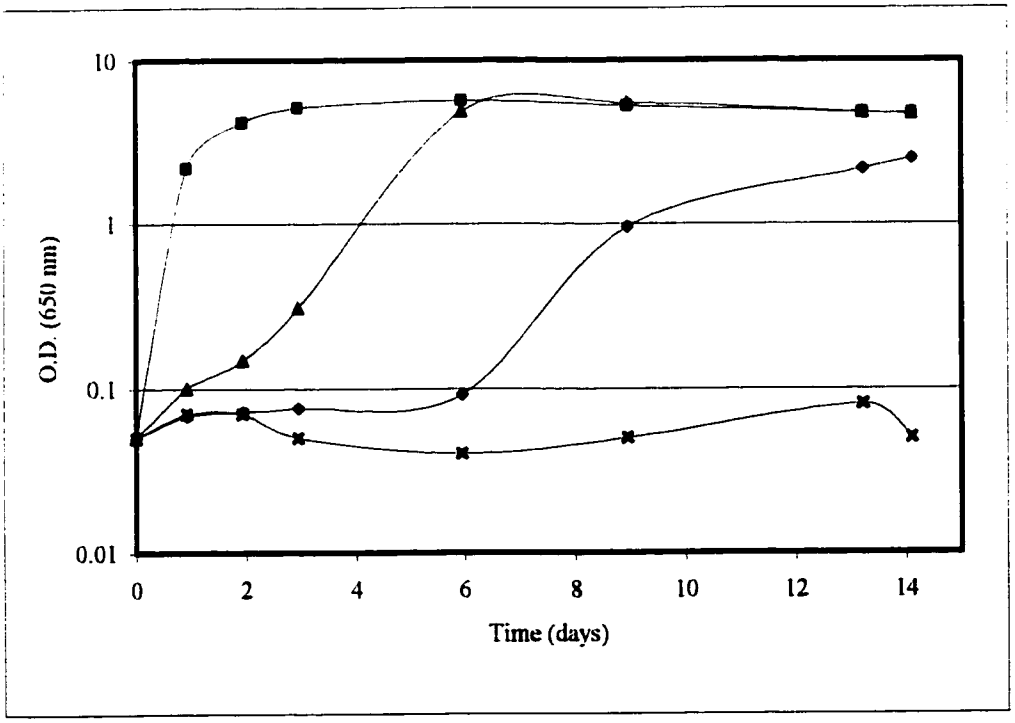
10
1
0.1
0.01
O.D. (650 nm)
0
2
4
6
8
10
12
14
Time (days)

Table 14: Generation times for cells grown on selected dihydric alcohols at 25°C.

Growth Substrate	Generation Time (h)
1,2-ethanediol	12.3
1,2-propanediol	3.7
1,3-propanediol	22.5
1,2-butanediol	21.1
1,3-butanediol	4.1
1,4-butanediol	21.6
2,3-butanediol	no growth
1,2-pentanediol	no growth
1,5-pentanediol	no growth
2,4-pentanediol	no growth
1,2-hexanediol	no growth

Figure 28: Ethylene glycol uptake with *P. fluorescens* grown on dihydric alcohols. Uptake was measured using ^{14}C-ethylene glycol. (ethylene glycol (◆), 1,2-propanediol (■), 1,3-propanediol (▲), 1,3-butanediol (✱), 1,4-butanediol (○))

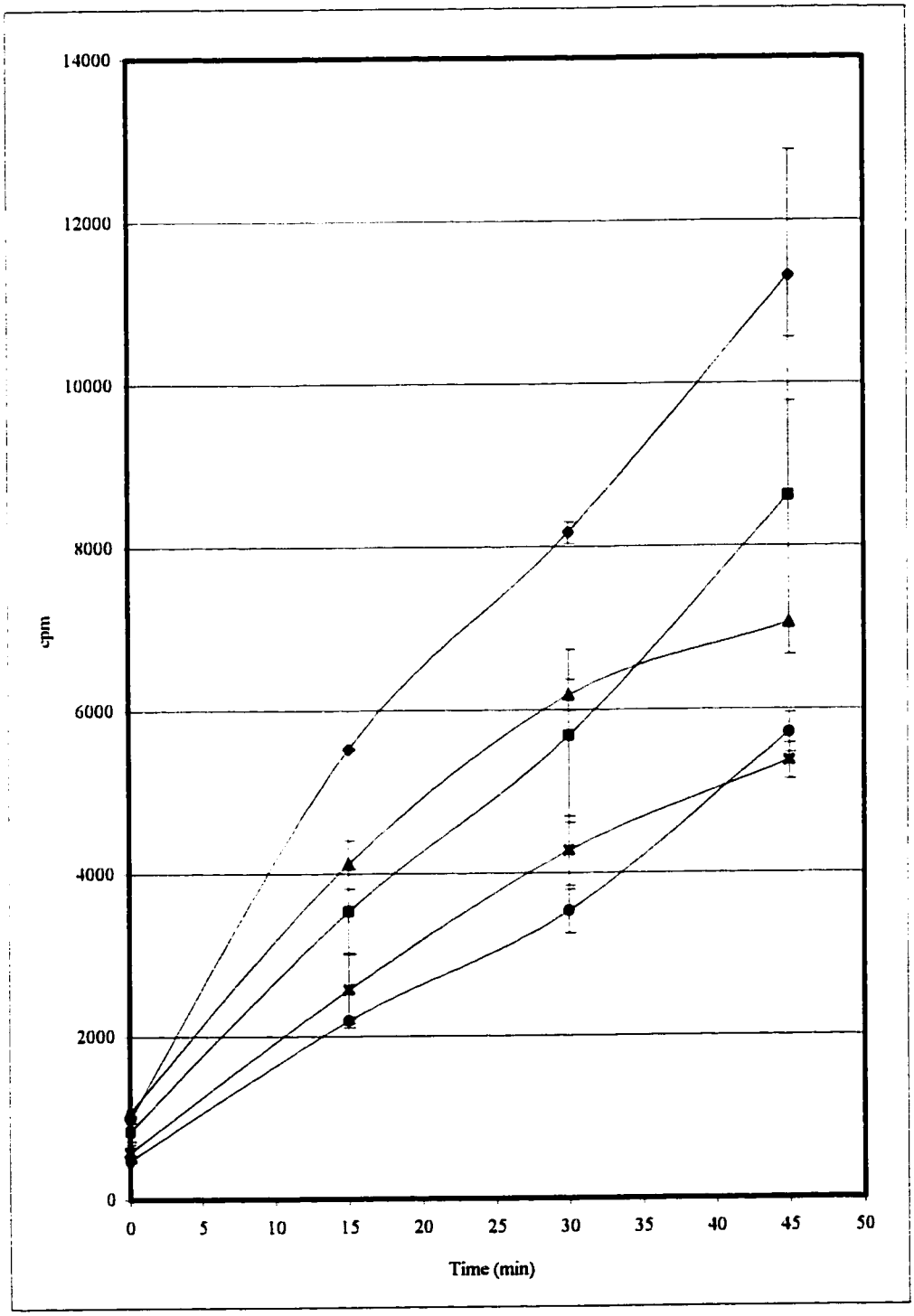
14000
12000
10000
8000
6000
4000
2000
0
cpm
0
5
10
15
20
25
30
35
40
45
50
Time (min)

Table 15: Induction of ^{14}C-ethylene glycol transport by growth on selected dihydric alcohols.

Growth Substrate	Uptake (cpm/min)	Relative Uptake (%)
1,2-ethanediol (control)	240 ± 3	100 ± 1
1,2-propanediol	163 ± 30	68 ± 13
1,3-propanediol	170 ± 8	71 ± 3
1,3-butanediol	123 ± 11	51 ± 5
1,4-butanediol	102 ± 10	43 ± 4

Figure 29: Competitive uptake with dihydric alcohols. Uptake was measured using ^{14}C-ethylene glycol. (no competitor added (◆), ethylene glycol added (■); Graph A: 1,2-propanediol (▲), 1,3-propanediol (✖); Graph B: 1,2-butanediol (▲), 1,3-butanediol (✖), 1,4-butanediol (○), 2,3-butanediol (●); Graph C: 1,2-pentanediol (▲), 1,5-pentanediol (✖), 2,4-pentanediol (○); Graph D: 1,2-hexanediol (▲))

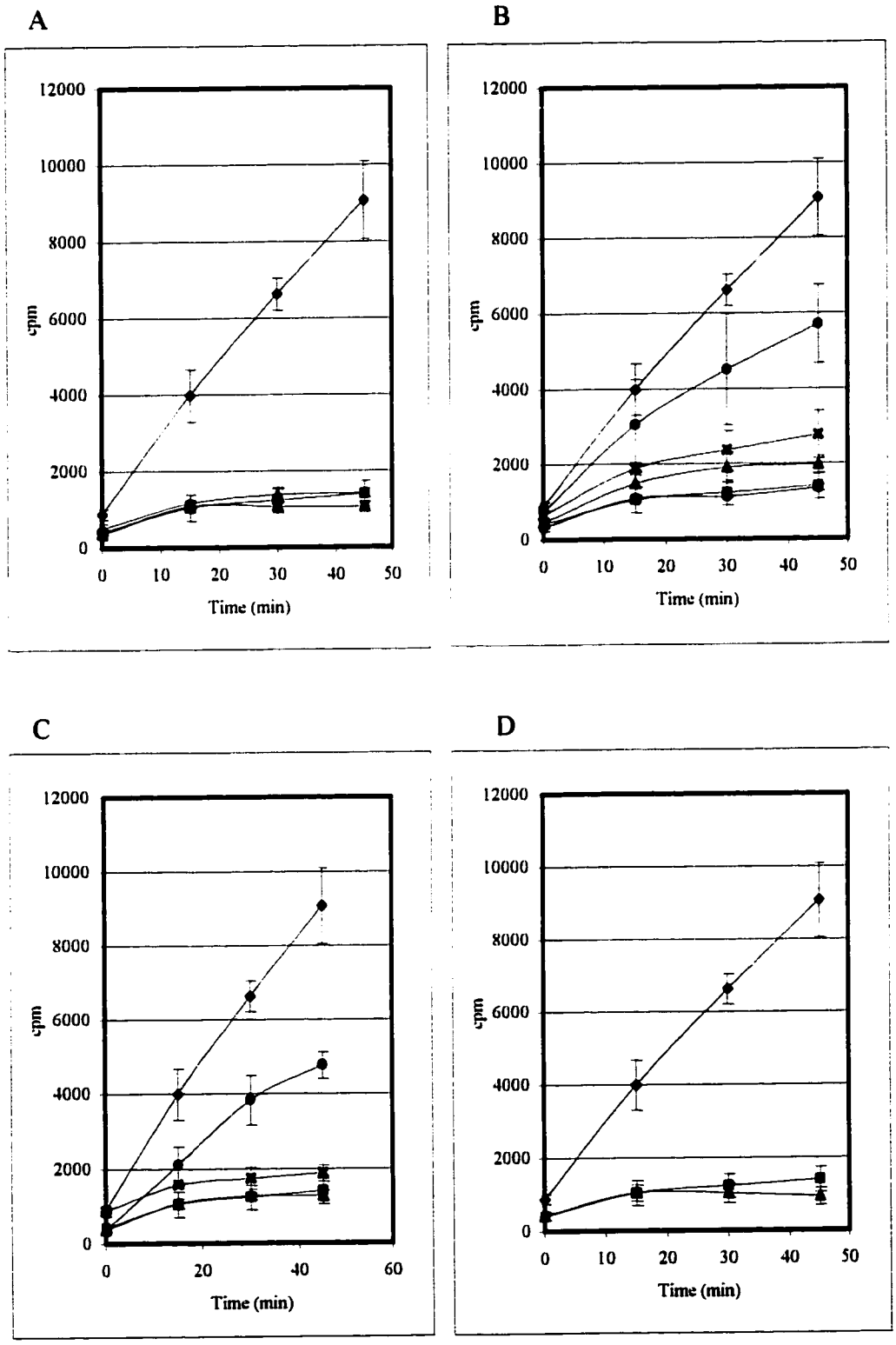
A
B
C
D
12000
10000
8000
6000
4000
2000
0
cpm
0
10
20
30
40
50
60
Time (min)

Table 16: Competition of dihydric alcohols with ethylene glycol during ^{14}C-ethylene glycol uptake.

Competitor	Uptake (cpm/min)	Relative Uptake (%)
no competition (control)	185 ± 26	100 ± 14
1,2-ethanediol	27 ± 8	15 ± 4
1,2-propanediol	29 ± 1	16 ± 0.5
1,3-propanediol	24 ± 0.3	13 ± 0.2
1,2-butanediol	49 ± 10	26 ± 5
1,3-butanediol	57 ± 12	31 ± 6
1,4-butanediol	27 ± 2	15 ± 1
2,3-butanediol	126 ± 41	68 ± 22
1,2-pentanediol	30 ± 4	16 ± 2
1,5-pentanediol	29 ± 9	16 ± 5
2,4-pentanediol	116 ± 24	63 ± 13
1,2-hexanediol	20 ± 5	11 ± 3

3.G.8. Effect of diethylene glycol upon ^{14}C-ethylene glycol transport

Diethylene glycol, a component of European deicing/anti-icing fluids, was investigated for its effect upon the transport process. The results for this experiment are presented in Figure 30. Although diethylene glycol cannot support growth of the microorganism, it did compete with the transport mechanism. The addition of diethylene glycol to the reaction vessel resulted in a 67% decrease in the relative uptake compared to the control (uptake dropped from 251 cpm/min to 79 cpm/min). This competition is also evident in Figure 31 where the presence of diethylene glycol negatively affected the growth of *P. fluorescens*. It would be expected that competition by a non-growth supporting substrate would decrease the observed growth and this is illustrated in Figure 31. However, diethylene glycol does not exhibit a higher affinity to the transport process than ethylene glycol. The addition of an equimolar concentration of nonradioactive ethylene glycol compared to diethylene glycol caused the relative uptake of ^{14}C-ethylene glycol to decrease to 14% (Fig. 30).

3.G.9. Effect of starvation upon ^{14}C-ethylene glycol transport

In a natural or wastewater treatment environment, the availability of ethylene glycol as a growth substrate will vary. There may be periods of time where the concentration of ethylene glycol is too low to support growth, resulting in a starvation scenario. The results for this starvation study (Fig. 32 and Table 17) showed that starvation did have a negative impact upon ethylene glycol uptake. Uptake rates after 1 day decreased by approximately 50%. After 7 and 15 days of incubation in the absence of a carbon source, the relative rates had decreased to 38% and 27%, respectively. Over the course of the starvation experiment, the optical densities at the various time points followed a similar decreasing trend. It is important to note, however, that the cell population, as determined through viable cell counts, remained fairly constant. A reduction in OD_{650} without a corresponding decrease in viability suggests that cell shrinkage was occurring and this physiological adaptation to starvation has a negative impact upon ethylene glycol uptake.

Figure 30: The effect of diethylene glycol on the uptake of ethylene glycol by *P. fluorescens*. Uptake was measured using ^{14}C-ethylene glycol. (no competition (■), ethylene glycol added (▲), diethylene glycol (◆))

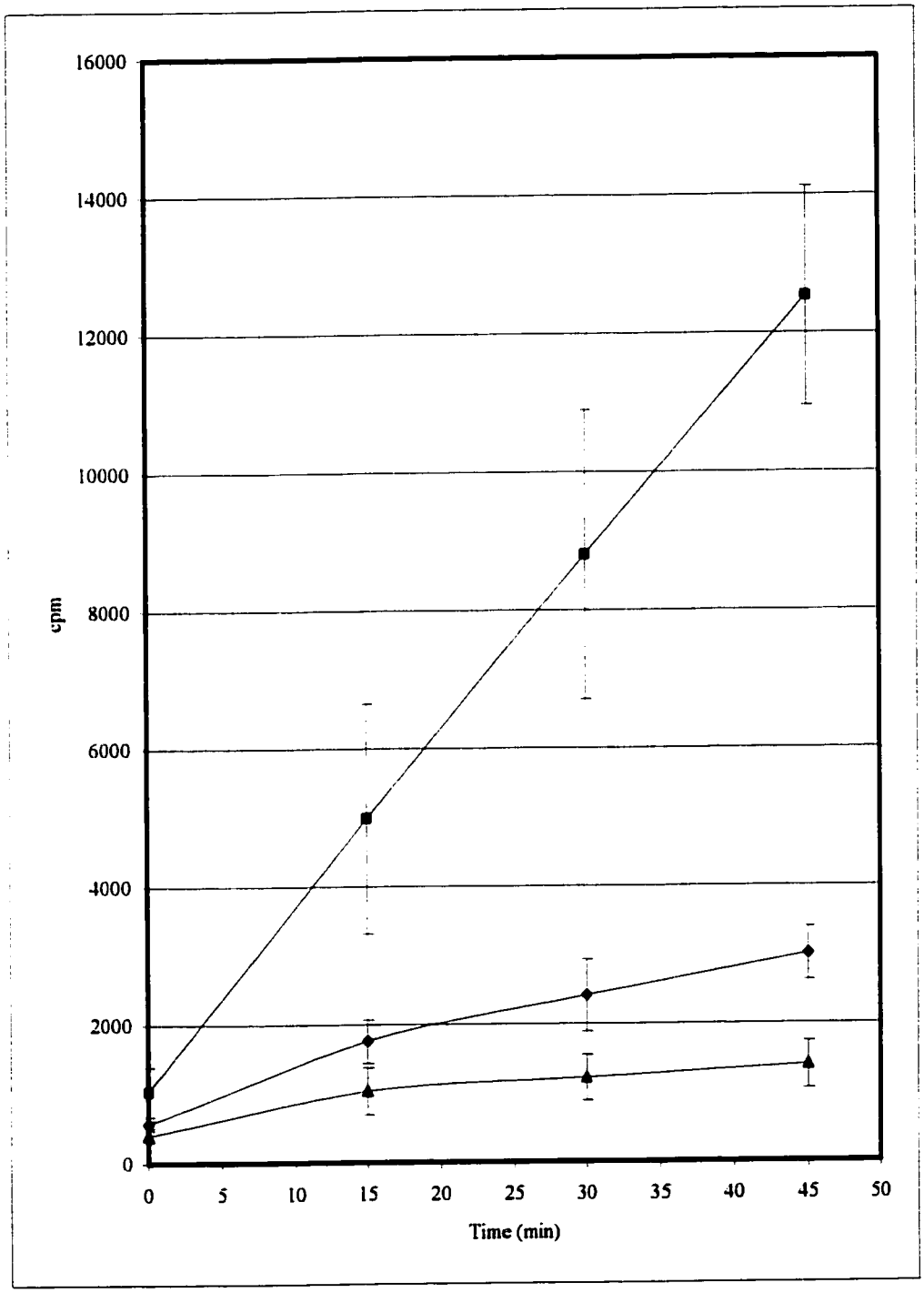
16000
14000
12000
10000
8000
6000
4000
2000
0
cpm
0
5
10
15
20
25
30
35
40
45
50
Time (min)

Figure 31: Growth of *P. fluorescens* in the presence of ethylene glycol and diethylene glycol. Growth was monitored by spectrophotometry. (ethylene glycol (◆), diethylene glycol (▲), 1:1 mix of ethylene glycol and diethylene glycol (■))

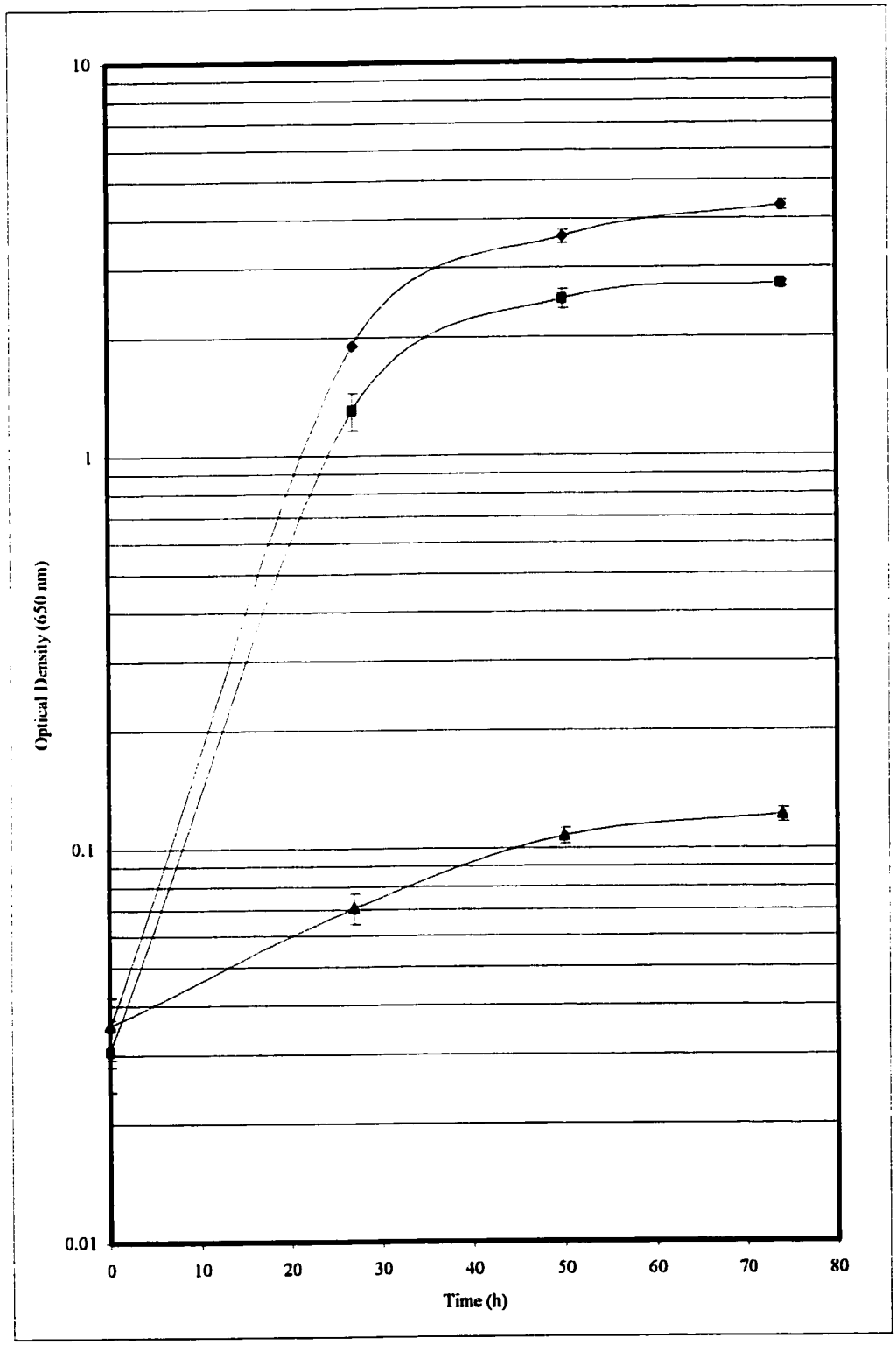
10
1
0.1
0.01
Optical Density (650 nm)
0
10
20
30
40
50
60
70
80
Time (h)

Figure 32: The effect of starvation upon ethylene glycol uptake. Uptake was measured using ^{14}C-ethylene glycol. (Starvation period: 0 day (◆), 1 day (■), 7 day (▲), 15 day (✱))

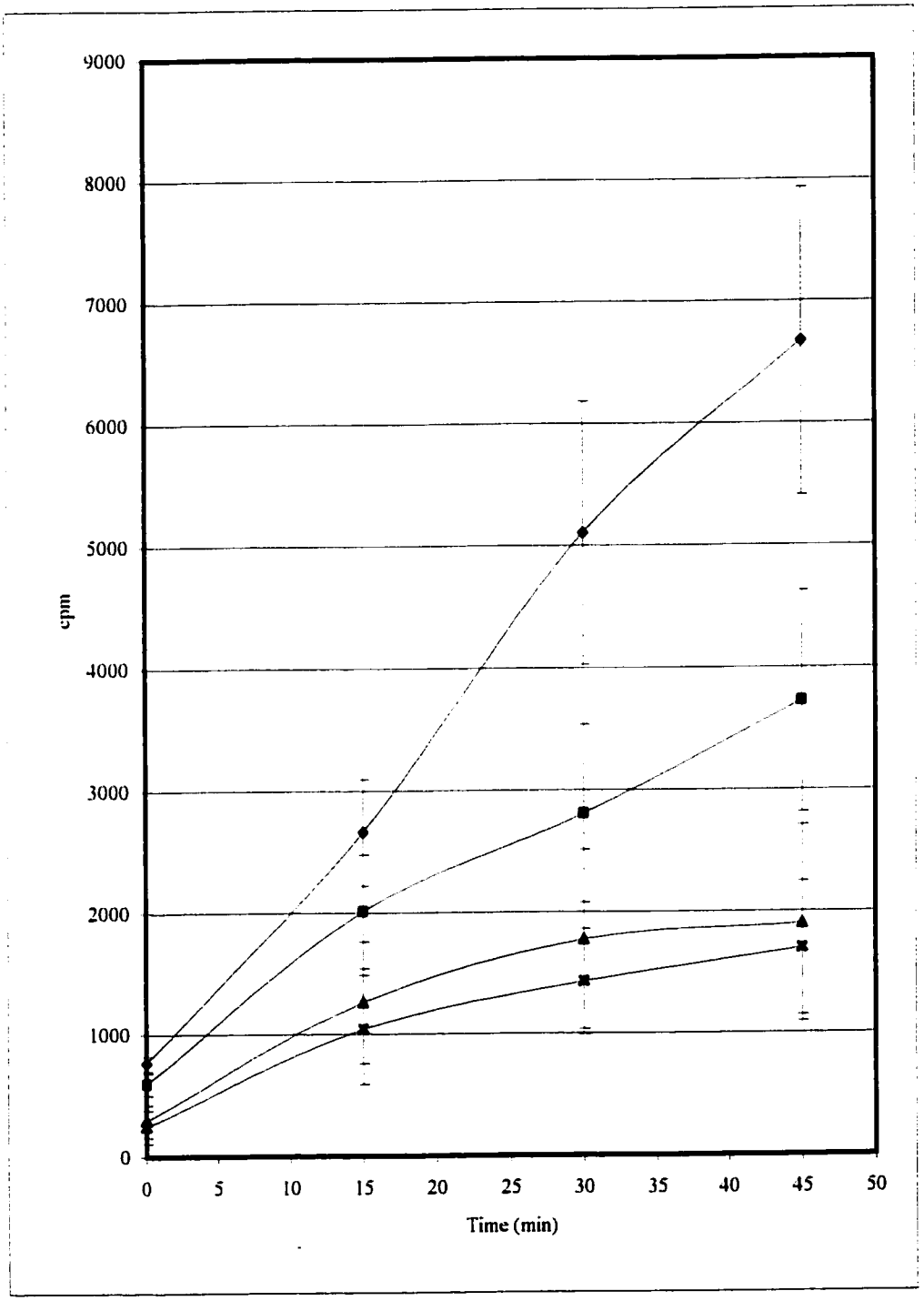
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
cpm
0
5
10
15
20
25
30
35
40
45
50
Time (min)

Table 17: Effect of starvation upon ^{14}C-ethylene glycol uptake.

Time of Starvation	Uptake (cpm/min)	Relative Uptake (%)	Optical Density	Cell Population (CFU/ml x10^9)
0 day (control)	145 ± 34	100 ± 23	1.01 ± 0.13	1.3 ± 5.0
1 day	74 ± 21	51 ± 14	0.92 ± 0.12	1.3 ± 0.0
7 days	55 ± 12	38 ± 8	0.62 ± 0.40	0.9 ± 0.1
15 days	39 ± 10	27 ± 7	0.52 ± 0.08	not analyzed

4. Discussion

The treatment of vast quantities of ethylene glycol waste produced by a variety of industries may benefit from options based on the degradative activities of microorganisms. The ability of the psychrotrophic *Pseudomonas fluorescens* used in this study to biodegrade ethylene glycol at temperatures ranging from 5°C to 25°C is an example of the degradative potential existing in the microbial world. In view of the need for economical treatment options for ethylene glycol waste, this microorganism was the focus of a study in which spent ethylene glycol-based deicing/anti-icing fluids originating from Pearson International Airport were treated at temperatures representative of those encountered during the winter season. In addition, a physiological approach was taken to investigate the factors affecting the transport of ethylene glycol into the cell. This type of information is useful in defining operational parameters in biotreatment schemes, especially if a particular variable, for example pH, is highly influential on the uptake of the substrate, consequently affecting the utilization observed.

Spent deicing/anti-icing fluids produced at many Canadian airports, including Pearson International Airport, are sent to municipal sewage treatment plants. The fees for this service are based on the biological oxygen demand (BOD) of the wastewater. In some locations, the spent fluids must be diluted such that the concentration of ethylene glycol being delivered is no greater than 50,000 mg/L (Strong-Gunderson *et al.*, 1995). In general, it costs approximately $3.75 for every litre of spent fluid disposed (Betts, 1999). Considering the volume of spent fluids collected in a single season, this amounts to a tremendous cost. Consequently, on-site treatment options are sought for. One potential option is biotreatment using psychrotrophic microorganisms. The fluids in question are used primarily at times of colder temperature; the resulting wastewater, which is stored in unheated underground tanks, has an average temperature below 10°C. The use of psychrotrophic microorganisms may be an economical biotreatment option considering that operation at ambient temperatures is possible, thus eliminating costs associated with heating.

The psychrotrophic *P. fluorescens* used in the current study may be one such organism with the potential to be used in the biotreatment of these spent fluids.

Growth of *P. fluorescens* was possible with either ethylene glycol or propylene glycol as the sole carbon source at both 25°C and 5°C. The growth rates on these carbon sources were quite similar with growth on propylene glycol being only marginally quicker at both temperatures. At the lower temperature, however, it was observed that cells acclimatized more rapidly to ethylene glycol compared to propylene glycol. Colucci and Inniss (1996) demonstrated, using 2-D SDS-PAGE, that upon switching the microorganism from glucose to ethylene glycol, proteins were induced. It was postulated that these proteins are related to the utilization of ethylene glycol (Colucci and Inniss, 1996). It appears that the same circumstance exists for propylene glycol. The presence of a lag period prior to growth on propylene glycol suggests that the pathway for propylene glycol metabolism is not continuously expressed and must be induced. These results are similar to those reported by Willetts (1979) whereby propylene glycol metabolism by a *Flavobacterium* was found to be an inducible process.

During growth on ethylene glycol and propylene glycol, the optical density of the cultures became high (O.D. > 5.0). For each experiment, the initial glycol concentration was in the range of 10,000 mg/L which is higher than most studies conducted with these compounds. The carbon:nitrogen:phosphorus (CNP; based on molarity) ratio was determined to be 100:8:14 for the ethylene glycol cultures and 100:7:12 for the propylene glycol cultures. It is generally accepted that the CNP ratio of 100:10:1 is optimal for substrate utilization and the ratios determined for the above experiments generally fall close to that target ratio. The higher phosphorus proportion was due to the fact that the growth medium utilized mono and dibasic potassium phosphate to provide the required buffering capacity. Although the CNP ratios for the growth experiments were close to that generally accepted as being optimal (i.e. 100:10:1), there was still the concern that the nitrogen and phosphorus concentrations in the media would eventually become limited, thus resulting in

cessation of degradative activity. To ensure this was not occurring, an enhanced medium was used which adjusted the CNP to 100:17:17 for ethylene glycol and 100:15:15 for propylene glycol.

The results of the utilization experiments indicated that the psychrotroph was capable of utilizing ethylene glycol and propylene glycol at both 25°C and 5°C. The use of an enhanced medium did not offer any advantage to substrate utilization; the standard basal salts medium was sufficient at providing necessary nutrient levels. At 25°C, the initial rates of utilization of ethylene and propylene glycol were similar with rates ranging from 2390 mg/L/day to 2650 mg/L/day. These rates are almost double those reported by Gonzalez *et al.* (1972) where a salt-requiring bacterium utilized ethylene glycol in liquid culture at a rate of 1376 mg/L/day over a temperature range of 24°C to 29°C and an initial ethylene glycol concentration of 8,000 mg/L. It was reported by Strong-Gunderson *et al.* (1995) that a microbial consortium consisting of Gram-negative rods was capable of ethylene glycol utilization in liquid culture at rates ranging from 1000 mg/L/day to 4000 mg/L/day at 25°C with an initial ethylene glycol concentration of 100,000 mg/L. In the current research, as well as the additional research cited above, the microorganisms being used are well adjusted to relatively high concentrations of ethylene glycol. In contrast, McGahey and Bouwer (1992) reported the successful utilization of ethylene glycol, however, their system was more sensitive to substrate concentration. A natural population originating from Mississippi soil was reported to be biodegradatively active at 100 and 1,000 mg/L of ethylene glycol but at concentrations equivalent to those reported above (i.e. 10,000 mg/L), there was little utilization observed.

At 5°C, the rate of ethylene glycol utilization (422 to 516 mg/L/day) was slightly faster compared to propylene glycol (349 to 355 mg/L/day). Unfortunately, due to the paucity of information regarding low temperature utilization of high concentrations of ethylene glycol and propylene glycol, comparisons to other findings are not feasible. Strong-Gunderson *et al.* (1995) mentioned that their microbial consortium was placed through

preliminary tests for activity at 4°C, however, no data were provided. In the current research, the initial rates of utilization were quite similar, but further examination indicates that complete removal of propylene glycol required considerably more time than that of ethylene glycol. The trend was observed at both 25°C and 5°C. Although ethylene and propylene glycol have similar structures, they are metabolized via different pathways (Willetts, 1979; Willetts, 1981). It would therefore not be unexpected for the microorganism to respond differently to the two substrates. The decrease in the rate of utilization of propylene glycol may be due to a number of reasons including the accumulation of inhibitory by-products as well as the depletion of dissolved oxygen in the culture flask. In a study conducted by Willetts (1979), it was determined that dissolved oxygen levels were a determining factor in the induction of two separate pathways for propylene glycol metabolism in a species of *Flavobacterium*. The existence of more than one metabolic pathway for a particular compound could potentially lead to more than one observable rate of utilization. The longer time required for removal may also indicate that propylene glycol is simply more recalcitrant compared to ethylene glycol as reported by Strong-Gunderson *et al.* (1995). It was found that although propylene glycol resulted in a higher optical density during growth, propylene glycol utilization was accompanied by a lower oxygen consumption and rate of removal compared to ethylene glycol. In the work reported by Gonzalez *et al.* (1972), cells grown on propylene glycol still exhibited a preference for ethylene glycol as the sole carbon source. Despite being acclimatized to propylene glycol, the cells oxidized ethylene glycol at a rate that was 4.5 times faster compared to propylene glycol.

For both ethylene glycol and propylene glycol, the initial reaction in aerobic utilization is the sequential oxidation of the terminal alcohol group via an aldehyde to a carboxylic acid. This reaction sequence is suspected to be carried out by microbial alcohol oxidases. For ethylene and propylene glycol, the oxidation sequence results in the production of glycolic acid and lactic acid, respectively. Although ethylene glycol and propylene glycol are similar in structure, oxygen consumption experiments suggested the

oxidases are likely specific for each substrate. Once glycolate and lactate are produced, they are metabolized by separate known pathways in the bacterial cell (Ornston and Ornston, 1969; Child and Willetts, 1978; Gonzalez *et al.*, 1972; Kornberg and Gotto, 1961; Morris, 1964; Stouthamer *et al.*, 1963, Willetts, 1979).

With the knowledge that ethylene glycol and propylene glycol are metabolized via different pathways, it was of interest to determine how the microorganism would respond if both compounds were present together in the same wastewater. With respect to growth, it was found that the generation times on the combined substrates was very similar to that observed on each individual carbon source. This was observed at both 25°C and 5°C. Furthermore, if the utilization rates for ethylene and propylene glycol are added together, this combined total is quite similar to the utilization rates for each substrate when used as the sole carbon source. A similar finding was reported by Klecka *et al.* (1993) where mixtures of deicing fluids (ethylene and propylene glycol based) were investigated for the removal of glycols. It was observed that the biodegradation of individual glycols was independent of whether the glycols were present singly or as a component of a mixture.

The above findings regarding ethylene glycol and propylene glycol degradation are supported in the literature (Fincher and Payne, 1962; Gonzalez *et al.*, 1972; Evans and David, 1974; Payne and Todd, 1966; Dwyer and Tiedje, 1983; Willetts, 1979). What was most significant about the current findings was that degradation was possible at the lower temperatures. It is this feature of the psychrotrophic *P. fluorescens* which makes it a possible candidate in the biotreatment of spent deicing/anti-icing fluids at ambient temperatures.

The deicing/anti-icing fluids used at Pearson International Airport Trillium Terminal 3 (Toronto, ON) are Union Carbide products called UCAR XL-54 (the deicer) and UCAR Ultra (the anti-icing fluid). In working with these fluids, it was necessary to determine whether the additives present in the formulations presented any effects upon the *P. fluorescens*. Although the exact composition of the fluids is proprietary information, it is generally known that the additives include a thickening agent, an emulsifier, a corrosion/rust

inhibitor and colouring. In addition, other compounds such as ethylene oxide, acetaldehyde, dioxane, high-molecular-weight polymers, polyamines, triazoles, urea, sodium nitrate, sodium benzoate, borax and benzotriazole may also be present as either impurities or additives (Fisher *et al.*, 1995). The presence of these other compounds supports the need to test deicing/anti-icing fluids for their toxicity as well as their susceptibility to biodegradation. This need is supported by a report in which ethylene glycol based deicing/anti-icing fluids were more acutely and chronically toxic to *Ceriodaphnia dubia* (the water flea) and *Pimephales promelas* (the fathead minnow) than pure ethylene glycol (Pillard, 1995).

It was found that growth was possible on the UCAR deicing/anti-icing fluids over the entire range of concentrations tested at 25°C. At the lower concentrations, there was little difference in growth between pure ethylene glycol and the deicing fluids; at concentrations greater than 50,000 mg/L, UCAR XL-54 imparted a negative affect in comparison to pure ethylene glycol whereas UCAR Ultra did not. It was found that at 100,000 mg/L, UCAR Ultra supported a higher rate of growth than the equivalent concentration of pure ethylene glycol. The reason for this observation is unclear. What was very significant about these findings was that degradation was possible at relatively high glycol concentrations. Most studies addressing deicing fluid biodegradation do not utilize concentrations as high as 10,000 mg/L. Of the studies available, the concentrations of deicing fluid tested ranged from 200 mg/L to 100,000 mg/L with the majority of the work focusing on concentrations below 5,000 mg/L (Sabeh and Narasiah, 1992; Klecka *et al.*, 1993; Strong-Gunderson *et al.*, 1995; Nitschke et at., 1996). The need to focus on microorganisms which are capable of withstanding and remaining biologically active at high glycol concentrations becomes evident when the concentrations in the collected spent fluids is taken into consideration. The collected spent fluids fluctuate with respect to their glycol content depending upon the deicing activities and weather conditions. With samples collected at Pearson International Airport, it was determined that ethylene glycol concentrations ranged from 500,000 mg/L in the high concentrate tanks to non-detectable levels in the underground low concentrate tanks.

Similarly, at Baltimore-Washington International Airport, ethylene glycol concentrations in the spent fluid samples ranged from non-detectable to 100,000 mg/L (Fisher *et al.*, 1995). Highest glycol concentrations are usually the result of increased deicing activities during periods of little or no precipitation. Although the high concentrations would still require dilution prior to treatment, the use of microorganisms capable of high concentrate activity would reduce the quantity of diluent required with a subsequent cost savings.

Experiments designed to test the growth and utilization of 10,000 mg/L (ethylene glycol w/v) unused UCAR deicing/anti-icing fluid at 5°C and 25°C were performed. The psychrotroph was indeed active at the low temperatures when using the deicing/anti-icing fluid as a substrate. The additives did not present any difficulties; in fact, the growth and utilization at 5°C were significantly higher than those observed with pure ethylene glycol. The generation times determined at 5°C were approximately half that on pure ethylene glycol and this was accompanied by an approximate 44% increase in glycol utilization rate. At 25°C, the growth was similar to that determined with pure ethylene glycol, however, the rates of glycol utilization were increased by 46%. These results are in contrast to those reported by Pillard (1995) where glycol-formulated deicers exhibit increased toxicity compared to pure glycols, however, Pillard's report does not utilize prokaryotic microorganisms. Unfortunately, a comparative toxicity study of formulated glycol deicers with reference to pure ethylene glycol for bacteria does not appear to be available. Worth noting here is that Strong-Gunderson *et al.* (1995) reported a similar scenario where it was observed that although a microbial consortium did not grow quite as strongly on a formulated ethylene glycol deicer, the rate of oxygen consumption was greater on the formulation compared to pure ethylene glycol. There was no explanation for this observation. The additives/impurities are obviously having an effect upon the microorganism. It is possible that certain compounds (i.e. urea or sodium nitrate) are stimulatory to the microorganism, thus leading to increased activity.

Of the handful of studies which investigate deicing fluid degradation, only a couple address biodegradation at low temperatures. Due to differences in the experimental procedure and reporting method, direct comparisons to the results in this thesis are difficult, however, general trends can be clearly identified. Sabeh and Narasiah (1992) investigated the biodegradation of UCAR ADF-D, an ethylene glycol-based formulation at 22°C, 10°C and 4°C. The mixed culture used in the study was capable of low temperature degradation; the rate of reaction was considerably reduced in comparison to 22°C. Klecka *et al.* (1993) investigated low temperature (-2°C and 8°C) biodegradation of various ethylene glycol and propylene glycol-based deicing fluids by a mixed culture in a soil environment. Once again, the rates of reaction reflected the low temperatures; it was concluded that in soil environments, biodegradation would still be a significant contributor to glycol removal during the winter months. Low temperature biodegradation of deicing fluids by a microbial consortium was also investigated by Strong-Gunderson *et al.* (1995) but the results were not reported.

An interesting item was that in the research reported by Sabeh and Narasiah (1992), it was determined that an organic load greater than 2.8 (food:microorganism ratio (F/M)) negatively affected the rate of reaction. It was also mentioned by Klecka *et al.* (1993) that organic load would influence the time required for complete biodegradation to occur. In the current research, the choice of 10,000 mg/L as an initial ethylene glycol concentration combined with an initial inoculum of approximately 5.7×10^7 CFU/ml suggests an F/M ratio of 185 (assuming average weight of one cell is 9.5×10^{-13} g; Neidhardt *et al.*, 1990); this F/M ratio is much higher than any tested in the literature. It is therefore quite significant that the *P. fluorescens* is capable of growth and utilization of the deicing/anti-icing fluids at 5°C even though the organic loading of the potentially toxic formulation is very high.

One of the main concerns regarding deicing/anti-icing fluids and the environment relates to the high oxygen demand that glycols place upon receiving waters. The carbonaceous 5 day BOD ($cBOD_5$) of pure ethylene glycol and propylene glycol has been

reported as 400,000-800,000 mg/L and 1,000,000 mg/L, respectively (Limno-Tech, Inc., 1999). Surface waters located near a number of major Canadian airports have been shown to contain ethylene glycol at concentrations ranging from <10 to 643 mg/L. This would translate into an oxygen demand of approximately 270 mg/L. At 20°C, the solubility of oxygen in water is about 9 mg/L, clearly not providing the necessary oxygen to support biodegradation. Excessive demands placed upon the dissolved oxygen in aquatic ecosystems can present a condition that is unfavourable to many aquatic organisms. This high oxygen demand can also influence the biodegradation of the substrates if the microorganisms responsible for utilization are affected. It was found that during ethylene glycol utilization, the dissolved oxygen decreased to less than 10% of complete O_2 saturation. This reduction in dissolved oxygen occurred despite the fact that the reaction vessel was aerated. Upon complete removal of the ethylene glycol, the dissolved oxygen levels in the reaction vessel returned to normal values. This observation suggested that *P. fluorescens* was capable of growth under or close to microaerophilic conditions. Similar findings were reported by Willetts (1981) in which a *Flavobacterium* was capable of oxidase-initiated ethylene glycol metabolism at oxygen concentrations as low as 7.75 mm Hg. Due to the low oxygen concentrations observed in the current research, it was of interest to determine whether the microorganism could utilize nitrate as an alternate electron acceptor. From the results, there was no advantage offered by the addition of nitrate to the media. The one exception was with UCAR Ultra at 5°C where the addition of nitrate resulted in a decrease in generation time. It was reported by Jank *et al.* (1974) that during the biodegradation of airport wastewater at low temperatures, there was an increase in the demand for nitrogen. As a result, the increased rate of growth in the UCAR Ultra-amended nitrate supplemented medium at 5°C may be a reflection of this finding.

With the knowledge that the psychrotrophic *P. fluorescens* was capable of utilizing relatively high concentrations of ethylene glycol based deicing fluids at 5°C, it was necessary to test this biodegradation with spent fluids. Spent deicing fluids are collected from the

tarmac via several different mechanisms (i.e. passive drainage systems, sweeper/vacuum vehicles). In addition to the components found in deicing fluids, the spent fluids contain precipitation (if any), fuels, oil and rubber products (Fisher *et al.*, 1995). Samples collected from Pearson International Airport had a distinct hydrocarbon odor.

Spent fluid samples were diluted such that the initial glycol concentration was approximately 10,000 mg/L. In addition to using BSM as a diluent, Borden groundwater was also utilized. The unsupplemented Borden groundwater was found not to support growth in any of the experiments. It was suspected that the unsupplemented Borden groundwater did not provide a sufficient nitrogen source. Therefore, in order to promote growth in the groundwater, a nitrogen and phosphate source were added. With respect to carbon:nitrogen:phosphorus, BSM and the supplemented Borden groundwater gave CNP ratios of 100:8:14 and 100:8:12, respectively. Both the supplemented Borden groundwater and BSM served equally well as a spent fluid diluent. However, using the supplemented Borden groundwater as a diluent provides a cost saving in that a defined medium is not required to support biodegradation.

Preliminary experiments conducted with the spent fluids indicated that a natural population was present and these microorganisms were capable of utilizing ethylene glycol. Analysis of the natural population indicated the presence of pseudomonads. The degradative abilities were investigated and it was found that the natural population was indeed active in the spent fluids when diluted to 10,000 mg/L. However, the rate of growth measured for the natural population was somewhat slower than that determined for the *P. fluorescens*. In conditions where both the natural population and *P. fluorescens* were present, it was observed that culture growth, in comparison to pure *P. fluorescens*, was negatively affected when BSM was used as a diluent. When the supplemented Borden groundwater was used, the culture growth rate at 25°C was very similar to that of a pure culture of *P. fluorescens*. In cultures where the natural population was eliminated, the *P. fluorescens* demonstrated rates of growth which were equal and greater than growth measured with pure ethylene glycol at

25°C and 5°C, respectively. An interesting observation was that at 5°C, only those cultures which received the psychrotrophic *P. fluorescens* demonstrated growth. Considering that deicing/anti-icing fluids are used during the winter and the ambient temperature is below 10°C, the natural population is not expected to contribute to any significant degree to the biodegradation of the target substrates. This fact illustrates the importance of psychrotrophs in low temperature bioremediation. However, it does not support the premise that psychrotrophs are absent in the natural population but rather suggests the absence of a combined psychrotrophic/ethylene glycol degradative ability within one species.

The utilization of ethylene glycol in the spent fluid experiments correlated closely with growth. Samples diluted with unsupplemented Borden groundwater did not utilize ethylene glycol. For those samples diluted with BSM or supplemented Borden groundwater, the rates of utilization at 25°C were quite similar to those on pure ethylene glycol with *P. fluorescens*. These results suggest that the removal of ethylene glycol from the spent fluids at 25°C does not require the addition of the *P. fluorescens*. All that seems to be required is dilution with a suitable diluent. However, at 5°C, the results confirmed the fact that the psychrotrophic *P. fluorescens* was required for utilization of ethylene glycol; at 5°C, the rate of glycol utilization was approximately 15% of that determined at 25°C. It was also observed that during the experiment, the hydrocarbon odor which was present in the spent fluids was reduced to the point where it was no longer detectable. Although a more thorough investigation is required to assess the ability of the microorganism to utilize the various hydrocarbons present in the spent fluid, something (i.e. biodegradation or volatilization) is happening to these compounds.

There are few studies investigating deicing/anti-icing fluids as microbial substrates; even fewer focus upon spent fluids. In 1974, Jank *et al.* reported on the ability of activated sludge to biodegrade airport wastewater containing spent deicing fluids. The experiments were conducted in bench-scale and pilot-scale reactor systems from which optimum loading conditions of effluent were obtained. Unfortunately, due to differences in experimental

design, it is difficult to compare the data obtained by Jank *et al.* (1974) to that obtained in the current research. In summary, it was determined that an organic loading of 0.15 kg BOD (kg MLSS day)$^{-1}$ was optimal at the temperatures tested (2, 5 and 10°C). Furthermore, increases in the organic loading led to an increase in the growth of filamentous microorganisms, thus limiting the activated sludge process. Strong-Gunderson (1995) also addressed spent deicing fluids for a formulation that was based on propylene glycol. It was determined that spent propylene glycol-based formulations were utilized by a microbial consortium consisting primarily of Gram-negative microorganisms; unfortunately, these studies were conducted at 25°C.

Degradation studies such as those presented above are very effective at demonstrating the abilities and limitations of a microorganism or microbial population at biodegrading a particular substrate. By using this information, parameters can be manipulated to obtain certain goals such as quicker removal rates. In addition to these types of studies, a physiological approach can also be invaluable. For example, biodegradative research may branch into enzymatic or genetic studies to further understand the processes at work. In the current research, ethylene glycol utilization by *P. fluorescens* was investigated at the level of substrate uptake. Such studies may lead to an understanding of how ethylene glycol enters the cell and what factors affect the rates of uptake observed. The ultimate goal would be to take the information obtained and apply it in the hope that enhanced biodegradation would result.

During all experiments dealing with ^{14}C-ethylene glycol uptake, the cultures used were grown, then harvested at very similar optical densities. This was to reduce the effect of culture age upon the rates of uptake. It was determined that over the course of microbial growth in batch culture, the rate of uptake varied. In general, maximal rates were recorded during early-exponential (OD_{650} = 0.28) and minimal rates were recorded during the decline phase (OD_{650} = 5.0). This observation is likely due to a combination of factors including pH, dissolved oxygen and toxic byproduct accumulation. Extremes in the external pH can have

serious consequences upon the pmf which subsequently affects the uptake of solute into the cell (Poolman *et al.*, 1987). Anoxic conditions may also affect transport by affecting electron transport chain activity. The reduced O_2 could hinder the proton pumping activities of the electron transport chain, thus affecting the overall pmf. The results demonstrated the need for consistency in preparing the uptake experiments. Variations in substrate uptake was also observed for *Alcaligenes denitrificans* BRI 3010 and BRI 6011 where maximal rates of benzoic acid uptake were noted in cells harvested during the late logarithmic phase of growth (Miguez *et al.*, 1995). However, the effects of culture age upon uptake may be substrate-related; the uptake of 2,4-dichlorobenzoic acid in *A. denitrificans* BRI 6011 was independent of culture age, despite the results for benzoic acid.

When *P. fluorescens* was shifted from glucose to ethylene glycol as the sole carbon source, a lag period occurred. After approximately 5 to 6 hours, the microorganism would resume growth at a rate which corresponded to the new environment. The mineralization of an organic compound is often preceeded by a lag period, commonly referred to an acclimation period. There are numerous reasons why a microorganism or a population of microorganisms would experience a stoppage in growth upon being switched to a new compound. The most common proposal is that the acclimation period is the result of the time required for the microorganism to synthesize the necessary enzymes corresponding to the new substrate. Related to this proposal is the theory that the lag phase corresponds to the time during which adaptive mutations or genetic exchange occurs. Additional explanations for the acclimation period include the preferential utilization of alternate carbon sources, the predation by protozoa upon the degradative microorganisms, the time required for the microorganism to acclimate to toxins or inhibitors as well as an insufficient supply of inorganic nutrients (Wiggins *et al.*, 1987). Since the acclimation in the current research is that of pure cultures in nutrient-sufficient media containing single carbon sources, the assumption is that the time required for resumption of growth is related to cellular adaptations.

When glucose grown cells were transferred to ethylene glycol as the sole carbon source, the microorganism experienced an acclimation period. Using 2-D SDS-PAGE, it was previously determined that *P. fluorescens* exhibited differences in the protein profiles when grown on glucose, glycolate or ethylene glycol. The protein profiles for the glycolate and ethylene glycol cells were very similar, supporting the hypothesis that ethylene glycol metabolism proceeds via glycolate (Colucci and Inniss, 1996). The assumption made was that the changes in the protein profiles for cultures grown on the various carbon sources was directly related to the induction/repression of necessary metabolic enzymes. For example, the initial oxidation of ethylene glycol is mediated by an alcohol oxidase which is inducible, as determined by oxygen consumption experiments in the current research.

In addition to the induction of metabolic enzymes, microorganisms are capable of inducing uptake mechanisms in response to new substrates. Inducible transport processes have been reported for a wide variety of microorganisms including *Alcaligenes denitrificans*, *Pseudomonas* sp. strain B13 and *Corynebacterium glutamicum* (Miguez *et al.*, 1995; Krämer *et al.*, 1990). The induction of the transport process may be a contributing factor to what defines the duration of an acclimation period. To further explore the physiological adaptations which *P. fluorescens* experiences during shifts in carbon sources, uptake studies with ^{14}C-labelled ethylene glycol were conducted. In addition, the uptake response to other environmental factors was also investigated.

For cells grown on glucose or glycolate, uptake of ^{14}C-ethylene glycol was not observed. In contrast, cells acclimated to ethylene glycol as a sole carbon source were quite active with respect to uptake of the radiolabel. These results suggest that ethylene glycol transport is an inducible process. The fact that glycolate does not induce ethylene glycol transport in an interesting fact considering glycolate and ethylene glycol are very similar in structure. It is possible that glycolate is transported by a specialized carboxylic acid transport system which is completely independent of ethylene glycol transport (Konings *et al.*, 1981). In fact, the transport of carboxylates by a specific group of transporters has been identified in

the major superfamily facilitator (MSF) grouping of secondary active transporters in *E. coli* (Pao *et al.*, 1998). Furthermore, it is generally accepted that transport proteins exhibit specificity for a specific range of substrates (Paulsen *et al.*, 1998). It was reported by Krämer *et al.* (1990) that *Corynebacterium glutamicum* harboured a highly specific transport system which recognized glutamate and aspartate as transportable substrates but none of the various glutamate analogues tested.

With the knowledge that solute transport is an inducible process, one can consider how this may affect the period required for acclimation to a new substrate. When a microorganism is transferred into a medium containing a different utilizable carbon source, the initial stages of adaptation likely involve the induction of the transport mechanism. Then, once the transport mechanism is active, the substrate can enter the cell and induce the synthesis of the necessary metabolic framework. Therefore, if the process of transport induction is slow, this step can greatly increase the time required for acclimation to the new carbon source. In a biodegradative process where the treatment of the waste does not follow a regular schedule (i.e. aircraft deicing), delays in the acclimation of a culture to the substrate can result in inefficient treatment. For example, at a municipal sewage treatment plant treating diethylene and propylene glycol waste, it was found that insufficient acclimation of the sewage bacteria to the glycols resulted in effluent containing detectable levels of diethylene glycol and elevated chemical oxygen demand (Nitschke *et al.*, 1996). Knowledge regarding the acclimation process may lead to enhanced methods of acclimatizing microorganisms to substrates.

The fact that ethylene glycol transport is inducible suggests that the process is mediated. In the simplest case, facilitated diffusion would provide a means by which the cell is able to acquire the necessary carbon source. In this process, stereospecific carriers are used to transport the solute across the membrane. However, while common in eucaryotic systems, facilitated transport is quite uncommon in procaryotes (Neidhardt *et al.*, 1990).

Instead, *P. fluorescens* possibly uses an active transport process to transport the glycols across the membrane.

Active transport is based on the chemiosmotic theory which explains the coupling between energy-generating and energy-consuming processes in the cytoplasmic membrane. This theory states that energy-transducing systems such as electron-transport systems and ATPase complexes act as proton pumps, translocating protons from the cytoplasm to the external medium. Since the cytoplasmic membrane is impermeable to ions, including protons and hydroxyl ions, the action of these pumps results in the formation of two energy-generating gradients. One gradient, known as the pH-gradient (ΔpH), forms as a result of the alkalinization of the cytoplasm and the acidification of the external medium. The second gradient, known as the electric potential ($\Delta\psi$), is formed by the removal of positive charges from the cytoplasm and the subsequent accumulation of these charges in the external medium. Together, these gradients form an inwardly directed force that is known as the proton-motive force (pmf) (Konings *et al.*, 1981; Simoni and Postma, 1975).

The coupling aspect of the chemiosmotic theory states that the pmf can be used to drive energy-consuming processes. These processes include maintaining cellular pH at the proper level, directing electrons through the respiratory chain in a reversed manner to reduce NAD when the supply of $NADH_2$ is low, driving ATP-synthesis through the use of ATPase, flagellar movement, and driving secondary active transport systems (Neidhardt *et al.*, 1990). It is the secondary active transport which is of interest in this research. Secondary transport systems have been demonstrated for a wide variety of solutes including amino acids, carboxylic acids, sugars, phosphorylated intermediates, inorganic cations and inorganic anions (Konings *et al.*, 1981).

Secondary transport of solutes can proceed via three general mechanisms termed uniport (one solute is translocated by the carrier protein), symport (two or more different solutes are translocated in the same direction by the carrier protein) and antiport (two or more different solutes are translocated by one carrier in opposite directions) (Konings *et al.*, 1981).

During symport transport, the solute (i.e. ethylene glycol) is mediated across the membrane together with a proton. Since the solute is neutral, the transport involves both a shift in charge and proton concentration meaning that both ΔpH and $\Delta\psi$ are involved in the transport process (Konings *et al.*, 1981). To maintain the pmf under these conditions, the cell must continue to pump protons from the cytoplasm to the external environment.

To test whether glycols are indeed utilizing the symport mechanism, two inhibitors were chosen based on their mode of action. The first inhibitor, 2,4-dinitrophenol (DNP), is a protonophore (uncoupler), which increases the membrane permeability for protons, ultimately dissipating the total pmf (Konings *et al.*, 1981). For the second inhibitor, dicyclohexylcarbodiimide (DCCD), the mode of action is through the inhibition of the F_0 part of the ATPase complex. The F_0 part, an assembly of 3 to 5 subunits, forms a channel that allows protons to pass through the cytoplasmic membrane. The inhibitor DCCD blocks this channel, essentially reducing the ability of the microorganism to counter the influx of protons during symport through ATP hydrolysis (Wilson and Smith, 1978; Konings *et al.*, 1981).

The results of the experiments using the above inhibitors support the theory that glycol transport is through the action of a symport process. It was found that DNP was very effective in reducing and completely inhibiting ^{14}C-ethylene glycol transport when added at 0.1 mM and 1.0 mM concentrations, respectively. The DNP likely eliminated the pmf thus resulting in the inability of the microorganism to operate a symport-based carrier. The action of DNP also supports the theory that ethylene glycol transport is not a passive process, due to the lack of transport in the absence of a pmf. The inhibition of ^{14}C-ethylene glycol transport in the presence of DCCD suggests that the process is sensitive to the disruption of cellular ATP concentrations. DCCD inhibits the formation of ATP, further supporting the theory that ethylene glycol transport is energy dependent.

Since the transport of ethylene glycol appears to be energy dependent through the action of a carrier, it was of interest to determine whether pH and temperature had any effect upon the behavior of the transport mechanism. The carriers which are utilized in secondary

active transport are transmembrane proteins and the question to be investigated was whether these proteins are susceptible to environmental factors in much the same way other proteins (enzymes) are.

It was determined that pH did have an effect upon the transport of ^{14}C-ethylene glycol across the cytoplasmic membrane. In fact, the effect correlates to the consideration that ethylene glycol transport is a secondary active transport process. The pH of the medium describes the proton concentration [H^+] and this ultimately has an effect upon the ΔpH and $\Delta\psi$ components of the pmf. In the experiments, it was determined that increasing the pH to 8 was strongly inhibitory to the uptake of ^{14}C-ethylene glycol. By increasing the pH to 8, the [H^+] concentration external to the cell was decreased leading to a decrease in the ΔpH and $\Delta\psi$ components of the pmf. With ethylene glycol transport apparently being linked to the pmf, this led to a decrease in the transport observed. In addition, alkalinization of the cytoplasm may also occur if homeostatic mechanisms such as the Na^+/H^+ antiporter are not operating or present (Krulwich *et al.*, 1990). By decreasing the pH to 6, there is an increase in [H^+], however, pH 6 is beyond the optimal pH growth range for *Pseudomonas fluorescens*. Although the pmf is stronger at pH 6, there may be other factors resulting in the decreased transport of ^{14}C-ethylene glycol observed. What was notable, however, was that the decrease in uptake at pH 6 was not as drastic as that observed for pH 8. This is important considering that during ethylene glycol utilization, the pH of the reaction vessel can decrease to as low as pH 5.3. Consequently, the tolerance to the lower pH compared to the higher pH is an advantage. Unless the pH is controlled, variations in pH will affect the microorganism by altering the secondary and tertiary structure and folding of proteins. For solutes that depend upon proteins for transport either directly (i.e. carrier proteins) or indirectly (i.e. ATPase), variations in pH are likely to influence the activities of those proteins. In the current research, the lower pH values tested (pH 5 and pH 4) likely resulted in structural modifications to the carrier which were sufficient to inhibit the transport of ^{14}C-ethylene glycol. From the results, it is clear that extremes in pH can affect substrate uptake; this will

ultimately affect degradation. It was reported by Fisher *et al.* (1995) that the pH in wastewater samples collected from an international airport ranged from pH 5.91 to pH 8.61. Having examined the effects of pH upon the uptake of ethylene glycol, it is now known that the pH of the spent fluid must be controlled for optimal utilization to occur.

Temperature was also found to affect the transport of ^{14}C-ethylene glycol. The temperature at which a microorganism is growing has a profound influence upon protein/enzyme mediated processes. Proteins exhibit variations in their three-dimensional configuration depending upon temperature. These changes ultimately lead to changes in function, and in the case of enzymes, rates of reaction. Since the transport of ethylene glycol is suspected to be mediated by a carrier (a protein), it was expected that a reduction in temperature would be accompanied by a decrease in transport, and this did in fact occur. At the higher temperatures, the uptake was relatively constant while at the lower temperatures, 5°C and 0°C, the uptake rates were more decreased. These results support the notion that the transport of ethylene glycol is an energy-dependent process. As the temperature is lowered, the energy required to fuel a certain enzyme mediated process increases (Feller *et al.*, 1996). Without an increase in energy input, the rate of reaction decreases and in the case of membrane transport, a decrease in solute uptake occurs. It was also noted by Nedwell and Rutter (1994) that as temperatures decrease, the specific affinity of a transport system for a particular solute also decreases. Reductions in the transport of solutes may also be a reflection of how membrane structure affects the functionality of membrane proteins. Temperature has a profound influence upon membrane structure and during a temperature decrease, membrane alterations occur in an effort to maintain a required level of membrane fluidity. The most common of the changes is the unsaturation of lipid acyl chains. In addition, modifications may also include a shortening of the average acyl chain length, an increase in the quantity of branched fatty acids as well as decreases in the proportion of cyclic fatty acids (Russell, 1984). These modifications may directly lead to changes in membrane protein activity, especially if the proteins require specific phospholipids in their

vicinity (In't Veld *et al.*, 1992). The activity of membrane-embedded proteins has also been shown to be sensitive to changes in membrane fluidity (Yuli *et al.*, 1981; Zheng *et al.*, 1988; Sikkema *et al.*, 1995). A combination of the above factors may account for the decreased uptake of ^{14}C-ethylene glycol at the lower temperatures. Regardless, uptake still occurred at the lower temperatures and this is significant given that the microorganism could potentially be used at ambient temperatures during winter deicing periods.

With the knowledge that ethylene glycol transport is an inducible process, it was of interest to investigate the ability of other similarly structured compounds (analogues) to induce and/or compete for the same transport mechanism. Ethylene glycol is a dihydric alcohol (Monick, 1968). When one hydroxyl group is involved in a reaction, dihydric alcohols exhibit a chemical behaviour similar to monohydric alcohols (i.e. ethanol) (Monick, 1968). It was of interest whether monohydric alcohols could induce and/or compete for the ethylene glycol transport mechanism.

Initial growth experiments demonstrated that *P. fluorescens* was capable of growth on primary alcohols (ethanol, 1-propanol, 1-butanol) but not on secondary alcohols (2-propanol, 2-butanol) or methanol. These results suggest that the microorganism produces an alcohol oxidase which is specific to primary alcohols. This hypothesis is supported by the knowledge that a primary alcohol oxidase catalyzes the initial oxidation of ethylene glycol to glycolate (Child and Willetts, 1978; Willetts, 1981). When cells acclimatized and grown on the growth-supporting monohydric alcohols were tested for their ability to induce the ethylene glycol transport mechanism, the results suggested that primary alcohols induced the system but the transport decreased with increasing carbon chain length of the inducing alcohol. This decrease in transport may be due to either a decreased induction of the transport mechanism or a decrease in the affinity of the induced mechanism to ethylene glycol.

Despite the fact that secondary alcohols were not growth supporting, it was observed that these compounds still competed for the ethylene glycol transport mechanism. In fact,

although the induction of the transport mechanism by primary alcohols was not very efficient, the competition by both primary and secondary alcohols with ^{14}C-ethylene glycol was very strong. This suggests that the transport mechanism is not highly specific towards glycols but rather recognizes a variety of similar molecules containing a hydroxyl group. Furthermore, it appears that the presence of the hydroxyl moiety takes precedence over its position within the structure. From this information, it can also be suggested that the inability to grow with secondary alcohols stems from a lack of necessary metabolic enzymes rather than the inability of the compound to be transported into the cell.

Dihydric alcohols were also tested for their ability to induce and/or compete with ^{14}C-ethylene glycol for the ethylene glycol transport mechanism. Initial growth experiments indicated that only those dihydric alcohols not exceeding 4 carbons in length were metabolized by *P. fluorescens* and, in addition, the molecule required the presence of a terminal or primary hydroxyl group. The specificity towards molecules containing a primary hydroxyl group was expected, given the results for monohydric alcohols. This specificity is likely due to the possession of a primary alcohol oxidase by the microorganism. Using the growth-supporting dihydric alcohols, cells were acclimatized to these compounds and subsequently tested for the induction of the ethylene glycol transport mechanism. As was observed for monohydric alcohols, increases in dihydric alcohol chain length were correlated with a decrease in transport efficiency. Once again, it is uncertain whether the decrease is due to reduced induction or a decrease in the affinity of the transport apparatus for ^{14}C-ethylene glycol. Regardless, induction is present and is most likely due to the presence of the hydroxyl moieties on the compounds.

The competition studies conducted with the dihydric alcohols suggest a certain specificity in the transport mechanism. Although the various pentanediol isomers tested were not growth-supporting, they did effectively compete with ^{14}C-ethylene glycol for transport. In addition, 1,2-hexanediol was also quite competitive. Although all of the dihydric alcohols tested were competitors for uptake, the presence of a terminal or primary

hydroxyl group provided a definite advantage. For the butanediol isomers, effective competition was shown for 1,2-, 1,3- and 1,4-butanediol whereas 2,3-butanediol was less effective. The same was observed for the pentanediol isomers where 2,4-pentanediol was less competitive than 1,2- and 1,5-pentanediol. While this decrease in competitiveness was not observed with secondary monohydric alcohols, the lack of a primary hydroxyl group in dihydric alcohols does influence the competitive character of the molecule. Although more work is required to elucidate the reasons for this, it may be related to the fact that dihydric alcohols contain two highly polar groups that more rigidly define molecular structure based on steric interactions.

P. fluorescens can not use diethylene glycol as a sole carbon source (Colucci and Inniss, 1996). Diethylene glycol is a diprimary glycol-ether, and is the simplest poly-form of ethylene glycol. Despite the simple structure, microorganisms generally have difficulty in the enzymatic cleavage of ether bonds (Kawai, 1987). The *P. fluorescens* in the present study does not have the enzymatic apparatus to cleave this bond thus making diethylene glycol a non-utilizable substrate. It was, however, still of interest to determine whether diethylene glycol would compete for the ethylene glycol transport mechanism. Diethylene glycol did compete for the transport mechanism likely due to similarities in its structure to ethylene glycol. The dihydric alcohol study indicated that the primary hydroxyl moiety has an influence upon uptake. Diethylene glycol possesses two such groups, although separated by an ether bond.

Due to the sporadic nature of deicing operations, the microorganisms being utilized in a biotreatment system for spent fluids may experience periods of little or no nutrient supply. Microorganisms being used in soil or aquatic environments to clean up glycol spills may also experience periods of decreased nutrient availability. Consequently, it was of interest to determine how starvation conditions would affect the transport of ^{14}C-ethylene glycol into *P. fluorescens*. During starvation, cells decrease RNA, protein and peptidoglycan biosynthesis while proteolysis and RNA degradation increase. This combination of events

leads to the formation of ultramicrocells, sometimes referred to as dwarf, mini, pico-, or nano-bacteria (Morita, 1988). Starvation may also result in changes to cellular membranes due to lipid utilization; how these changes affect membrane function is not entirely clear (Hood *et al.*, 1986; Morita, 1988). When cells experience starvation conditions and undergo the various physiological changes, they are in a state of starvation arrest. However, the entire population may not attain this physiological state. In one study by Kurath and Morita (1983), it was determined that after 18 days starvation, only 1% of the bacteria present were respiring and viable. Conversely, in a study by Van Overbeek *et al.* (1995), it was found that after 72 days starvation, a population of *P. fluorescens* was still greater than 90% viable. Viability following extended periods of starvation has also been demonstrated in the marine psychrophilic *Vibrio* ANT-300 (Novitsky and Morita, 1977) and *Vibrio cholerae* (Hood *et al.*, 1986). In the current study, after 7 days, the population of viable *P. fluorescens* cells remained relatively constant. At the same time, however, the optical density of the culture dropped considerably suggesting that under starvation conditions, the size of these cells decreased, yet the majority remained culturable. Van Overbeek *et al.* (1995) reported a similar observation where *P. fluorescens* decreased in size by 51% after only 5 days starvation.

Despite the fact that the starved cell population remained relatively constant, substrate uptake decreased by 64% after 15 days. Under starvation conditions, the cell attempts to maintain ATP levels (Kurath and Morita, 1983). Considering that 15 to 20% of the cellular ATP is used for active transport processes (Kurath and Morita, 1983), it is important that this pool be available in the event that nutrients become available. Exactly how the cell maintains this ATP pool in the absence of exogenous substrates is unclear but it is generally accepted that it is partially derived from the utilization of internal carbon sources including accumulated carbohydrates, poly-β-hydroxybutyrate, proteins and lipids (Morita, 1988). Kurath and Morita (1983) demonstrated that a marine *Pseudomonas* sp. was capable of maintaining its ATP content following a brief initial decrease. This was accompanied by a

similar response in substrate uptake as measured by ^{14}C-glucose and ^{14}C-glutamic acid. Upon starvation, an initial decrease in the uptake of these compounds was observed, however, the levels of uptake eventually returned to normal values and in the case of glucose, the rate of uptake increased three-fold. Thus it appears that this particular microorganism is demonstrating the ability to maintain substrate uptake over the course of starvation. The fact that *P. fluorescens* in the current study is not capable of maintaining uptake during starvation may be the result of an inability to maintain a sufficient ATP pool. Alternatively, the reduction in uptake may be due to the proteolysis of the ethylene glycol transport proteins in an effort to supply the cell with necessary energy for survival. Reductions in uptake of mannitol were reported for a *Pseudomonas* sp. after only 30 h of starvation; the transport system could be easily induced upon the addition of exogenous mannitol (Morita, 1988). Difficulties in transport may also arise due to the membrane alterations which occur as a result of lipid utilization (Sikkema *et al.*, 1995). Unfortunately, information in the area of starvation and membrane alteration/function is limited.

In summary, the psychrotrophic *P. fluorescens* used in this study was able to grow upon and utilize at both 25°C and 5°C ethylene glycol, propylene glycol and Union Carbide ethylene glycol-based UCAR XL-54 and UCAR Ultra. The growth of the microorganism when using the deicing/anti-icing fluids was not unlike that observed with equivalent concentrations of pure ethylene glycol. In fact, the utilization of the ethylene glycol in the formulations was faster than that with pure ethylene glycol. These results suggest that the additives present in the deicing/anti-icing formulations are not inhibitory at the concentrations used (formulation diluted to 10,000 mg/L ethylene glycol).

The spent deicing fluids collected at Pearson International Airport were readily biodegraded at rates comparable to pure ethylene glycol degradation at both 25°C and 5°C. The natural population of bacteria found in the spent fluids was capable of ethylene glycol degradation, however, this ability was restricted to 25°C. The addition of the psychrotroph

was required to achieve glycol removal at 5°C. It was also determined that inorganic nutrients were required to promote biodegradation in the diluted spent fluids.

The initial oxidation of ethylene glycol and propylene glycol appeared to be catalyzed by separate oxidases. However, growth on propylene glycol did induce the energy dependent transport mechanism responsible for ethylene glycol uptake. It was also determined that structurally similar compounds were capable of inducing and competing for the ethylene glycol transport mechanism to varying degrees.

The transport mechanism was affected by pH, temperature, culture age and nutrient limitation (starvation). This suggests that during biodegradation, it may be necessary to maintain certain environmental/culture conditions to maximize substrate utilization.

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Appendix

Figure 33: Aerobic and anaerobic pathway of ethylene glycol metabolism. (Adapted from Willetts, 1981)

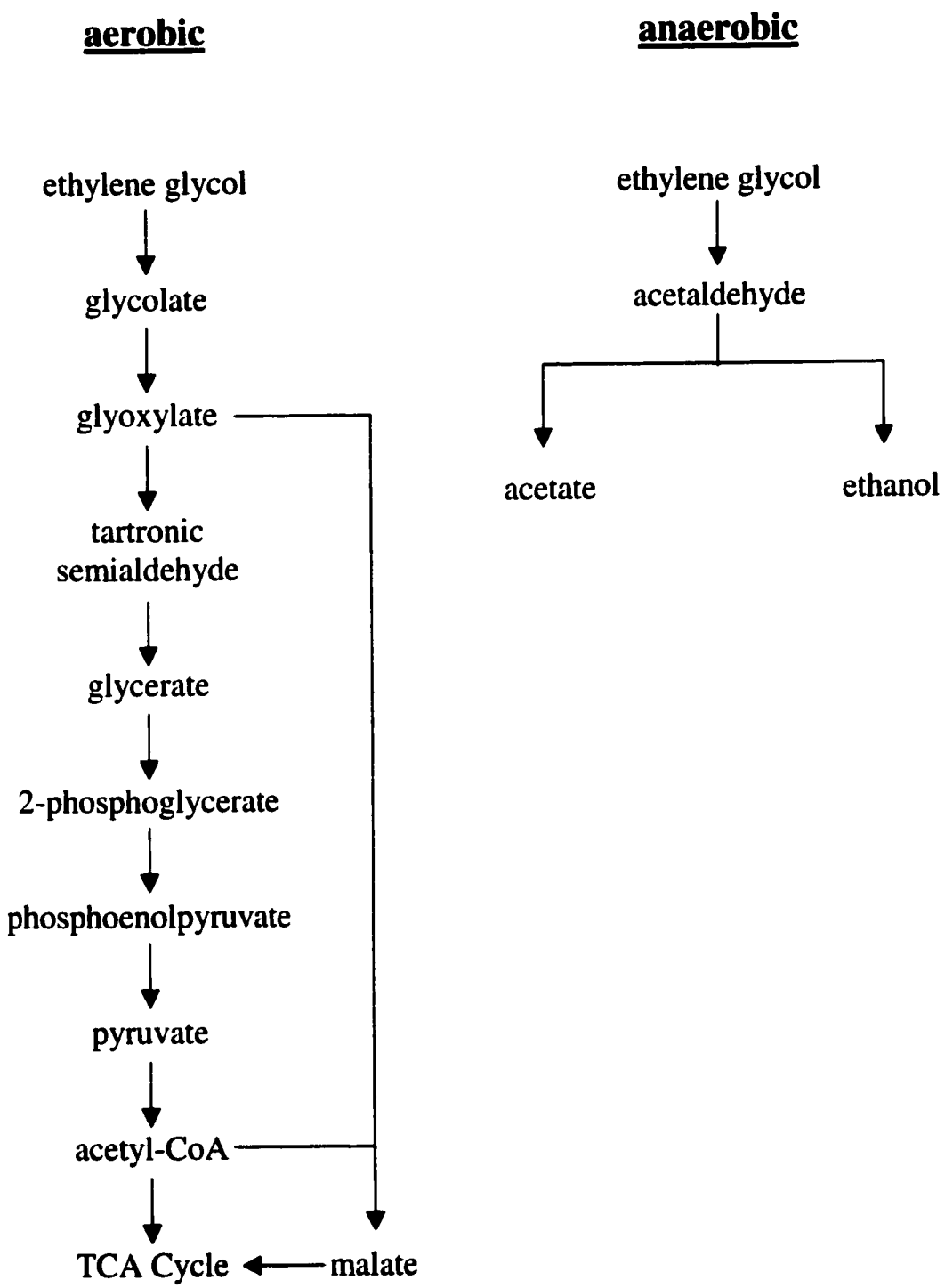
aerobic
anaerobic
ethylene glycol
glycolate
glyoxylate
tartronic semialdehyde
glycerate
2-phosphoglycerate
phosphoenolpyruvate
pyruvate
acetyl-CoA
TCA Cycle
malate
ethylene glycol
acetaldehyde
acetate
ethanol

Figure 34: Aerobic and anaerobic pathway of propylene glycol metabolism. (Adapted from Willetts, 1979)

aerobic

propylene glycol

↓

lactaldehyde

↓

lactate

↓

pyruvate

↓

acetyl-CoA

↓

TCA Cycle

anaerobic

propylene glycol

↓

propionaldehyde

↓

n-propanol