

# Timed Sampling Experimental Process to Assess Carbon-14 Consumption by Microbes



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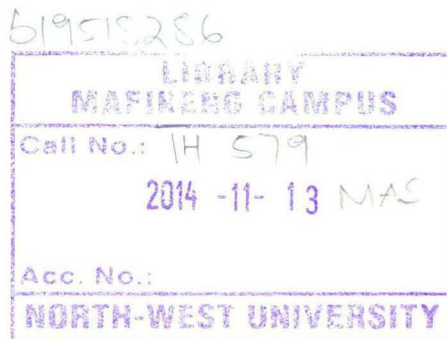
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Dissertation submitted to the faculty of agriculture, science and technology in partial fulfilment of the requirements for the degree Master of Science in Applied Radiation Science and Technology at the Mafikeng campus of the North West University

Supervisor: Dr. M.L. Dunzik-Gougar

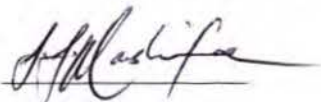
Co-Supervisor: Dr. P.J. Williams



November 2011

# DECLARATION

I, the undersigned, Itumeleng Ignatious Mashifane, hereby declare that the work presented in this dissertation except where otherwise indicated, is my own original work and has not been submitted to any university for purposes of obtaining a degree.

A handwritten signature in black ink, appearing to read 'I.I. Mashifane', written over a horizontal line.

I.I. Mashifane

November 2011

# ABSTRACT

Carbon-14 ( $^{14}\text{C}$ ) in large concentrations can be a hazard to living things due to its long radioactive half-life, and the ease with which it can enter the biosphere. During the operation of high temperature gas-cooled reactors (such as the Pebble Bed Modular Reactor)  $^{14}\text{C}$  is created in the graphite components. The Waste Minimization Project of PBMR (Pty) Ltd. is investigating methods of  $^{14}\text{C}$  removal from graphite to allow simplified disposal or reuse of this high quality nuclear grade material.

The work summarized in this thesis represents preliminary steps taken to assess the feasibility of microbial remediation of irradiated graphite. Scoping experiments were designed and performed to establish baseline conditions for future experiments using irradiated graphite. Experiment objectives were to (1) test the compatibility of candidate bacteria with a graphite system, (2) identify the bacterial growth medium optimum for the system, and (3) create a bioreactor environment in which bacteria consume a source of surrogate graphite  $^{14}\text{C}$ .

To achieve the first two objectives, a selection of five single bacterial species and a mixed bacteria culture were introduced separately to media containing glucose and/or graphite as the carbon source. Daily monitoring of bacterial growth in the bioreactors was accomplished via measurement of visible light absorbency and/or pH of the liquid medium. The measured values indicated that for all species growth was least in the systems with graphite and no glucose. However, it was unclear if graphite had the expected negative effect on growth when glucose was present. On average, the most significant bacterial growth occurred in the systems containing the mixed culture, as opposed to the single species. This preliminary screening resulted in the choice of the mixed bacteria culture and Štyriaková growth medium (with at least partial glucose) for use in subsequent experiments.

To address the third objective, bioreactor media were supplemented with one or two carbon containing salts (sodium acetate,  $\text{NaC}_2\text{H}_3\text{O}_2$ , and sodium bicarbonate,  $\text{NaHCO}_3$ ) chosen to represent the possible chemical bonding type of  $^{14}\text{C}$  in irradiated graphite. To increase the probability of bacterial consumption of this alternate carbon

source, less of the usual food source (glucose) was provided. In the preliminary salt experiments, non-active carbon salts were used and bacterial growth monitored to assess the effect of the salts at various concentrations. Light absorbance and pH values indicated the best bacterial growth occurred in the bioreactor containing a mixture of the two salts with  $\text{NaC}_2\text{H}_3\text{O}_2$  as the larger component. Further, it was concluded that glucose (0.12 M), as a carbon source together with fractions of the sodium salts, should be included in the growth medium.

While bacterial growth did occur in the presence of the carbon containing salts, there was no direct evidence that showed that the salts had been metabolized. To determine the extent, if any, to which the bacteria processed this glucose alternative carbon source,  $^{14}\text{C}$ -labelled  $\text{NaC}_2\text{H}_3\text{O}_2$  and/or  $\text{NaHCO}_3$  were introduced. Carbon-free-air transferred the gas produced in the bioreactors to a tube furnace employed to oxidise carbon-containing gases to carbon dioxide ( $\text{CO}_2$ ). The oxidised gas was subsequently trapped in a NaOH solution. Identical bioreactors were configured and operated for different periods of time in order to assess the evolution of the bacterial growth process. At the end of reactor operation, solid (bacterial biomass), liquid and gas phase samples were collected for analysis via Liquid Scintillation Counting (LSC).

Results of liquid scintillation analysis indicate the bacteria did metabolize  $^{14}\text{C}$  from the dissolved salts. At the beginning of reactor operation all  $^{14}\text{C}$  was in the liquid phase. However, after operation  $^{14}\text{C}$  was distributed among the three phases. Location of  $^{14}\text{C}$  in the solid biophase is particularly suggestive of bacteria metabolizing of the carbon-containing salts. Significant differences in  $^{14}\text{C}$  distribution were noted between the sodium acetate and  $\text{NaHCO}_3$  systems. Radioactivity of bacterial biomass from the acetate salt system indicated as much as half of the  $^{14}\text{C}$  was incorporated in this phase. In contrast, there was no significant activity detected in the bicarbonate system biomass phase. This difference in activity is consistent with the observed growth difference between the two non-active salt systems. The gas phase of both salt systems contained significant quantities of  $^{14}\text{C}$  (30%-60% of system inventory). The expected source of  $^{14}\text{C}$ -containing gas in the  $\text{NaHCO}_3$  system is chemical decomposition of the bicarbonate anion in solution. In the  $\text{NaC}_2\text{H}_3\text{O}_2$  system, the presence of gas phase  $^{14}\text{C}$  is consistent with the bacterial

processing indicated by solid phase  $^{14}\text{C}$  content; however, there may also be chemical degradation of the acetate anion in solution.

The overall results of these experiments indicate microbial remediation may be a suitable treatment option for irradiated graphite from nuclear reactors. The chemical form of  $^{14}\text{C}$  in irradiated graphite may be a determining factor in the ultimate feasibility of such an application. Further testing with irradiated graphite is necessary to determine the chemical form of  $^{14}\text{C}$  and the viability of bacterial processing of that chemical in the radioactive environment.

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# LIST OF ABBREVIATIONS

AVR	Arbeitsgemeinschaft Versuchsreaktor
Bq	Becquerel
Bq.g <sup>-1</sup>	Becquerel per gram
Bq.ml <sup>-1</sup>	Becquerel per millilitre
<sup>12</sup> C	Carbon-12
<sup>13</sup> C	Carbon-13
<sup>14</sup> C	Carbon-14
CO <sub>2</sub>	Carbon Dioxide
CO	Carbon Monoxide
<sup>60</sup> Co	Cobalt-60
CPM	Counts per minute
°C	Degrees Celsius
DPM	Disintegrations per minute
dH <sub>2</sub> O	Distilled water
g	Gram
g.l <sup>-1</sup>	Gram per litre
HLW	High Level Waste
HTGR	High Temperature Gas cooled Reactor
Hr	Hour
<sup>3</sup> H	Hydrogen-3 (Tritium)
ILW	Intermediate Level Waste
IAEA	International Atomic Energy Agency
LLW	Low Level Waste
LSC	Liquid Scintillation Counting
L	Litre
L.h <sup>-1</sup>	Litre per hour
CH <sub>4</sub>	Methane
μL	Micro Litre
ml	Millilitre
min	Minutes

M	Molar
nm	Nanometres
<sup>14</sup> N	Nitrogen-14
NECSA	Nuclear Energy Corporation of South Africa
<sup>17</sup> O	Oxygen-17
PBMR	Pebble Bed Modular Reactor
PBMR (Pty) Ltd	Pebble Bed Modular Reactor (Proprietary) Limited
PBR	Pebble Bed Reactor
<sup>40</sup> K	Potassium-40
pH	Potential of Hydrogen
QIP	Quench Indicator Parameter
rpm	Revolutions per minute
NaCH <sub>3</sub> CO <sub>2</sub>	Sodium Acetate
NaHCO <sub>3</sub>	Sodium Bicarbonate
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
t-SIE	Transformed Spectral Index of the External standard
TRISO	Tri-structural Isotropic coated particle
<sup>235</sup> U	Uranium-235
<sup>238</sup> U	Uranium-238
UO <sub>2</sub>	Uranium Oxide
UF <sub>6</sub>	Uranium Hexafluoride
H <sub>2</sub> O	Water

# **1. INTRODUCTION**

## **1.1 General overview of PBMR**

The world's economy depends on the availability of energy, and this increases the consumption and demands for electricity, a preferred end-use form of energy for both developing and industrialized countries (Semenov et al., 1991). There have been many alternative energy sources (like solar, wind, biomass, geothermal and tidal power) that were thought to meet the growing demands. However, because they were not practically available, proven, and economically competitive compared to large-scale conventional energy sources such as coal, oil, gas, hydropower and nuclear energies (Semenov et al., 1991), they were deemed as additional energy sources to supply the base-load during off peak hours.

The world has united in the pursuit of reducing the carbon dioxide and greenhouse emissions during electricity generation. In comparison to the above mentioned power sources, nuclear power is seen as one of the most feasible sources now available to generate electricity in the quantities needed and without producing greenhouse gases (Semenov et al., 1991). In supporting this quest, South Africa is developing its first high temperature reactor in the form of the Pebble Bed Modular Reactor (PBMR) (Nichols, 1998).

PBMR (Pty) Ltd. is a South African company designing the PBMR, a Generation IV nuclear reactor that would use small tennis-ball-sized fuel spheres (pebbles) to provide a low power density reactor. The pebble bed concept was adopted from the German's Arbeitsgemeinschaft Versuchsreaktor (AVR) research reactor that operated for 20 years (Matzner, 2004). Low power density and a large graphite core are key factors contributing to the inherent safety of this design. Even under conditions of accidental loss of the gas coolant, graphite efficiently conducts heat away from the fuel, preventing it from melting (Matzner, 2004). This type of passive cooling is significant improvement

over active emergency core cooling system required in today's light water cooled reactors (LWRs).

The PBMR fuel particles as depicted in Figure 1.1, consists of a uranium oxide ( $UO_2$ ) kernel (<1 mm) that is isotropically triple coated with porous carbon, pyrocarbon and silicon carbide, respectively (Matzner, 2004). These coatings provide a good barrier against the release of fission products from the fuel kernel. The TRi-ISOtropic coated (TRISO) particles are embedded in a graphite matrix material that is formed into a sphere, or pebble, about 6.0 cm in diameter (Sawa and Minota, 1999).

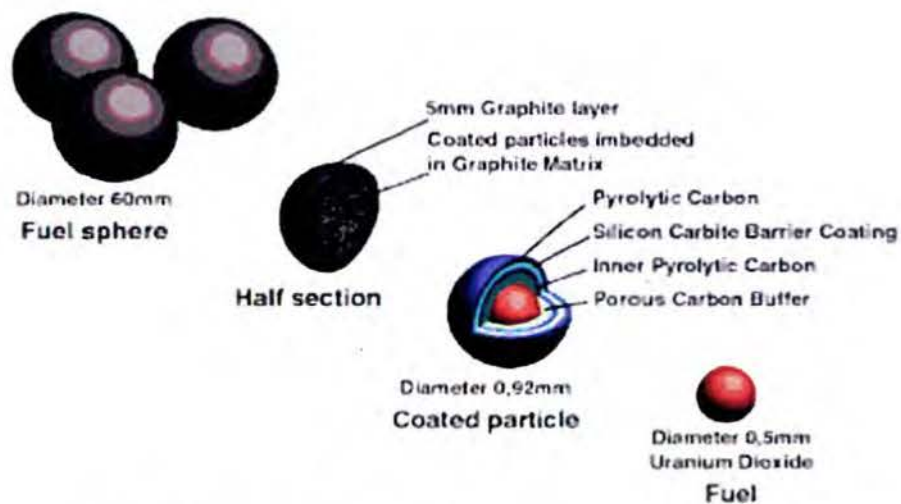


Figure 1.1: Fuel pebble and microspheres (Matzner, 2004)

The used PBMR fuel pebbles presents waste management challenges different than those created by ceramic  $UO_2$  pellets used in the Generation 1 and 2, LWRs. The ceramic fuel pellets are relatively small in volume, but high in radioactivity, while PBMR fuel spheres are relatively low activity, high volume waste due to the matrix graphite. In addition to graphite in the fuel pebbles, large structural components of the reactor core are made of graphite and must be disposed of at the end of reactor life (Dunzik-Gougar et al., 2008).

The PBMR (Pty) Ltd Waste Minimization Project is investigating methods for waste volume reduction and recovery. One scenario for irradiated graphite remediation includes a two step approach, beginning with removal of non-carbon radionuclides (activation products, fission products and actinides) from the bulk material on an elemental basis. This process may be chemical, microbial or biochemical in nature. Secondly,  $^{14}\text{C}$  produced in graphite during irradiation requires separation at an isotopic level. PBMR is studying methods of treatment using biological processes found in natural systems. The technology is based on using a consortium of radiation tolerant microorganisms to rid the graphite of radionuclides (Dunzik-Gougar et al., 2008).

## **1.2 Problem statement**

During the lifetime of a PBMR, large volumes of fuel matrix and structural graphite are irradiated. This material, while high in volume, is low in specific radioactivity. To minimize the amount of waste produced by this Generation IV reactor, irradiated graphite clean-up and recycle are being considered. A radionuclide of particular interest in the graphite is  $^{14}\text{C}$ , a long-lived (5730-year half-life) isotope of carbon. As a low-energy  $\beta$ -emitter,  $^{14}\text{C}$  does not present an external radiation hazard; however, because of its long half-life and potential for incorporation into carbon-based life, it is considered problematic for long-term waste disposal. Isolating  $^{14}\text{C}$  from the bulk waste and separately immobilizing it would minimize the potential hazards (IAEA, 1999). Microbial treatment of the radioactive graphite may be an efficient method of  $^{14}\text{C}$  removal resulting in lower volume radioactive waste and possible recycle of the high quality graphite.

### 1.3 Objectives

The work reported in this thesis is part of larger project with the overall objective to determine the feasibility of microbial processing of  $^{14}\text{C}$  in irradiated graphite. The experiments performed were designed to establish baseline conditions for future experiments using irradiated graphite samples. Objectives of these scoping experiments were to (1) test the compatibility of candidate bacteria with a graphite system, (2) identify the medium optimum for bacterial growth, and (3) create a bioreactor environment in which bacteria consume a source of surrogate graphite  $^{14}\text{C}$ .

## 2. LITERATURE REVIEW

### 2.1 Radioactive waste

As with all types of power production, nuclear power generation creates waste. Radioactive waste from nuclear power is unique in that it is low volume compared to other types of power generation, such as gas emissions and ash from coal power. Also unique is the fact that the composition of radioactive waste changes over time via radioactive decay. All radionuclides decay and eventually produce a stable, non-radioactive species. This process may take less than a second or more than a million years. To protect living things from the radiation released, radioactive waste is isolated or diluted. Radioactive waste often is classified as shown in Table 2.1.

Table 2.1: Classification of radioactive waste (IAEA, 1994)

Waste type	Method of shielding	Description of component
Exempt	Hazards are negligible, no shielding required	Negligible amounts of radioactivity and may be disposed of with domestic refuse
Low-Level Waste (LLW)	Shielding not required for handling and transportations	Paper, rags, tools, clothing, and filters, which contain small amounts of mostly short lived radioactivity
Intermediate-Level Waste (ILW)	Barriers of lead, concrete or water	Resins, chemical sludges and metal fuel cladding, as well as contaminated materials from reactor decommissioning
High-Level Waste (HLW)	Barriers of lead, concrete or water	Fission products and transuranic elements generated in the reactor core

Apart from the commercial nuclear power industry, there are other entities, like government defence and research organizations, academia and medical institutions that produce radioactive wastes; however, the vast majority of waste, by volume and activity, is produced by nuclear power.

### **2.1.1 PBMR graphite waste**

In a PBMR, like most High Temperature Gas-cooled Reactors (HTGR), graphite is used in structural components and as a fuel matrix material. Nuclear grade graphite is made from good quality crystalline graphite and amorphous carbon, and has various structural matrices (Takahashi et al., 1992). A report from Bushuev et al. (1992) suggests that graphite has an open pore structure, with a total porosity of about 26%. Compositions of nuclear grade graphites are similar to that of commercially available non-nuclear grade graphite; however, the fraction of impurities in new nuclear grade material is less than that of graphites in reactors currently operating.

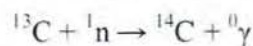
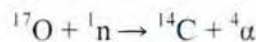
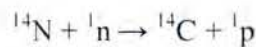
During irradiation in a nuclear reactor, graphite becomes radioactive due to the neutron activation of impurities. There are two groups of radioisotopes contained in irradiated graphite, the short-lived isotopes such as  $^{60}\text{Co}$ , and the long-lived isotopes, principally  $^{14}\text{C}$ . The former group makes graphite difficult to handle, but decays quickly after tens of years, while the latter group is of concern during decommissioning because of its long half life and the possibility of it being discharged into the biosphere (Mason and Bradbury, 1992).

### 2.1.2 Carbon-14 in irradiated graphite

A radionuclide of particular interest in the graphite is  $^{14}\text{C}$ , a long-lived (5730-year half-life) isotope of carbon. As a low-energy  $\beta$ -emitter,  $^{14}\text{C}$  does not present an external radiation hazard; however, because of its long half-life and potential for incorporation into carbon-based life, it is considered problematic for long-term waste disposal.

It is expected that the chemical form of  $^{14}\text{C}$  in irradiated graphite is not crystalline graphite, but rather some more reactive form. The exact nature of  $^{14}\text{C}$  chemical form(s) is unknown and it being characterised by PBMR and other entities (Dunzik-Gougar et al., 2008). Studies of irradiated graphite, as summarized by Marsden et al. (2002), suggest  $^{14}\text{C}$  bonding with some combination of N, O, H and C. All of these species are present and available in the graphite structure during irradiation. Bonding with these elements is also consistent with the location of  $^{14}\text{C}$  in the graphite outer surface, where open pores may contain water and nitrogen impurities from air exposure.

Production of  $^{14}\text{C}$  in HTGRs is via neutron activation of (1)  $^{14}\text{N}$  in graphite pores and coolant gas, (2)  $^{17}\text{O}$  in the  $\text{UO}_2$  kernels of the fuel spheres and oxygen-containing impurities in the graphite, and (3)  $^{13}\text{C}$ , which comprises about 1% of natural (elemental) carbon. These activation reactions are represented in equation form as follows:



The predominant source of  $^{14}\text{C}$  in the PBMR waste is the neutron activation nitrogen (Khripunov et al., 2008).  $^{14}\text{C}$  quantities produced from  $^{17}\text{O}$  and  $^{13}\text{C}$  reactions are deemed negligible, because of the limited quantities of each isotope (0.04%  $^{17}\text{O}$  and 1%  $^{13}\text{C}$ ) and their relatively small thermal neutron absorption cross-section (0.24 barns for  $^{17}\text{O}$  and 1.4 millibarns for  $^{13}\text{C}$ ) (Khripunov et al., 2008). In contrast, 99.6% of nitrogen present in the graphite is  $^{14}\text{N}$ , which has a thermal neutron absorption cross section of approximately 2

barns. Takahashi et al. (1992) suggest that newly formed  $^{14}\text{C}$  atom remain at the same location of formation, which likely is the location of the  $^{14}\text{N}$  precursor diatomic molecule.

## **2.2 Treatment and disposal of radioactive graphite**

Graphite has been part of the past and present reactor designs being used as a neutron moderator and reflector and as a fuel matrix material. Countries around the world are considering disposal options as they cope with existing inventories of irradiated graphite and plan for future power plants. The main disposal options were summarized by the International Atomic Energy Agency (1999): (1) direct disposal after suitable packaging, 2) disposal after incineration with consequent ash conditioning, and 3) disposal after chemical treatment and conditioning, and proper packaging. Although relatively small amounts of  $^{14}\text{C}$  make it to the environment, the overall inventory of this radioisotope continues to grow as the need for power production increases. Therefore, long-term options are needed for its management (Yim and Caron, 2006).

### **2.2.1 Treatment of PBMR graphite**

Preliminary plans for lifetime operations of the PBMR call for on-site storage of spent pebble fuel for the reactor's operating period. Before being accepted at a disposal site, both the pebbles and large graphite structural components would need pre-treatment according to the levels of radioactivity. Additional options considered for PBMR spent fuel include spent fuel deconsolidation, matrix graphite reclamation and storage of coated particles. On-site facilities would be used for the removal of matrix material and of outer pyrolytic graphite from the coated particles. Separated graphite would be treated to remove  $^{14}\text{C}$ , stored and later reused for other applications. Waste generated during the separation and cleaning processes is to be solidified and treated as HLW (Yim and Caron, 2006).

The Waste Minimization Project of PBMR (Pty) Ltd. focuses on research and development of methods for removal of  $^{14}\text{C}$  from irradiated graphite. A review of general separation options is presented in the next subsection.

## 2.2.2 Elemental vs. isotopic separation

Elemental separation of non-carbon radioisotopes from irradiated graphite has been used over the years to treat the radioactive waste. There are techniques (ion exchange, chemical oxidation, adsorption and membrane processes) that have been used for the separation or removal of specific radioactive elements from mostly aqueous solutions. These include removal of soluble organics and separation of radioisotopes from water, soil or gas samples. Biological processes exist that are based on the principle of emulating natural occurring processes that have been used to treat the waste. The techniques use microorganisms that have evolved to survive in hostile environments and adapt to changes in the environment (Battista, 1997; White et al., 1999).

There are isotope separation techniques that separate isotopes based on their mass. The separation techniques include diffusion and centrifuge, electromagnetic, laser, and chemical methods. The most noted of the mentioned methods is the technique used on uranium enrichment for nuclear reactor fuel production and weapons material. Natural uranium (99.3%  $^{238}\text{U}$  and 0.7%  $^{235}\text{U}$ ) is reacted to form uranium hexafluoride gas ( $\text{UF}_6$ ). The mass difference between  $^{235}\text{UF}_6$  and  $^{238}\text{UF}_6$  allows gradual separation of the two forms over many stages of diffusion or centrifuge cascades (Cochran and Tsoufanidis, 1999).

Isotopic level separation is not really necessary for removing the  $^{14}\text{C}$  created from  $^{14}\text{N}$ , because it can be removed chemically, i.e. it's a different chemical form than the bulk  $^{12}\text{C}$ . PBMR's Waste Minimization Project has focused on bioremediation in its initial studies. The studies were based on the elemental separation of  $^{14}\text{C}$  using microbial remediation.

## **2.3 Microbiology of radionuclide bioremediation**

### **2.3.1 Bioremediation of contaminated environments**

Mueller (1996) defined bioremediation as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities. According to Vidali (2001), bioremediation uses naturally occurring bacteria, fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment. These microorganisms transform contaminated compounds through reactions that take place as part of their metabolism. The bioremediation technique has largely been overlooked for industrial application; however, as technology improves, there is new focus on bioremediation as a low-cost and low technology option with a positive public image.

Biotransformation, bioaccumulation and biosorption are different mechanisms that have been observed in various bacteria, and could be applied in the remediation of radioactive waste (Sara and Sleytr, 2000; Burke *et al.*, 2005).

#### **2.3.1.1 Biotransformation**

Microbes have subjected radionuclides such as U, Tc and Cr, in enzymatic detoxification. The oxidised forms of these metals (U, Tc and Cr) are highly soluble in aqueous media and mobile in groundwater, whereas reduced forms are insoluble and precipitable into the solution (Macaskie, 2000). Also reported (Suzuki *et al.*, 2003; Brodie *et al.*, 2006) is the extracellular precipitation of metals and radionuclides by *Desulfovibrio desulfuricans*, *Geothrix fermentans*, *Deltaproteobacteria* sp. and *Clostridium* sp.

Lloyd and his colleagues (2003) have made major advances to understand the reduction mechanisms of Fe(III), U(VI) and Tc(VII) by *Geobacter sulfurreducens*. The researchers discovered that the surface-bound *c*-type cytochrome (9.6 kDa), which is present on the periplasmic surface of *G. sulfurreducens*, is required for U(VI) reduction, while the cytochrome *c*-deficient mutant of *G. sulfurreducens* was found to be inefficient to detoxify U(VI). Also found to be detoxified by different mechanisms using a periplasmic Ni/Fe-containing hydrogenase enzyme of *Desulfovibrio fructosovorans*, that uses hydrogen as the electron donor for the metal reduction process, was the Tc(VII) metal (De Luca *et al.*, 2001). Additionally, the efficient indirect mechanisms could also be significant in the immobilisation of Tc(VII) in the sediments when biologically reduced forms of Fe(II) or U(IV) transfer the electrons to Tc(VII) directly.

There are various bacteria that are able to reduce highly soluble chromate ion to Cr(III), which then precipitates as Cr(OH)<sub>3</sub>. Other Cr(IV)-reducing microbes such as *Arthrobacter aurescens*, *Pseudomonas aeruginosa*, *Pantoea agglomerans* and *Desulfovibrio vulgaris*, have been isolated from chromate-contaminated water, oils and sediments (Ganguli and Tripathi, 2002; Arias and Tebo, 2003; Goulhen *et al.*, 2006; Horton *et al.*, 2006).

### 2.3.1.2 Bioaccumulation and biosorption

Solutes are transported during bioaccumulation, from the outside of the microbial cell through the cellular membrane then into the cytoplasm. By using the bioaccumulation mechanism, various microbes, such as *Micrococcus luteus*, *Arthrobacter nicotianae*, *Bacillus megaterium* and *Citrobacter* sp., have been implemented for the bioremediation of radioactive waste materials (Macaskie *et al.*, 2000; Tsuruta, 2003).

Douglas and Beveridge (1998) defined biosorption as an association of soluble substances with the cell surface. This technique arises from the presence of various ionisable groups present in the lipopolysaccharides (LPS) of Gram-negative, as well as

the peptidoglycan, teichuronic acids and teichoic acids of Gram-positive bacterial cell walls. The (bacterial) cell walls may also be over-layered by various surface structures, which are able to interact with metal ions. These may be composed primarily of carbohydrate polymers (capsules) or proteinaceous surface layers (S-layers) (Douglas and Beveridge, 1998). Uranium is reported (Merroun *et al.*, 2005) to be accumulated by *Bacillus sphaericus*, through cells containing an S-layer.

### 2.3.1.3 Biostimulation and bioaugmentation

North and his colleagues (North *et al.*, 2004) describes biostimulation as the addition of nutrients (carbon and other essential nutrients), and this serves to increase the number or activity of indigenous microflora available for bioremediation activity. Biostimulation of U(VI) immobilisation is one of the promising strategies for *in situ* remediation of U(VI). The *in situ* immobilisation of uranium via the microbial reduction of soluble U(VI) to insoluble U(IV) can be used to prevent the migration of uranium in groundwater (Vrionis *et al.*, 2005). The immobilisation processing of uranium remediation is accelerated by the addition of acetate to a uranium-contaminated aquifer, which effectively stimulates the growth of dissimilatory metal-reducing microorganisms belonging to the family of Geobacteraceae (Ortiz-Bernad *et al.*, 2004).

The process of adding microorganisms that are able to transform or degrade contaminants is known as bioaugmentation. The added microorganisms can either be a new species or mixed microbial communities existing on the sites of contamination (Leung, 2004). *Dehalococcoides ethenogenes* has been successfully introduced, as a small obligate anaerobe, into the subsurface for an extended time period to reductively dechlorinate tetrachloroethylene to ethylene (Holmes *et al.*, 2006). Similarly, the nonstop addition of microorganisms to a reactor site in the ground or using *ex situ* treatment of contaminated groundwater can improve radionuclide bioremediation. Microorganisms cultured in the laboratory or produced in on-site bioreactors may also be employed in *ex situ* treatments or injected back into the subsurface for *in situ* treatment.

### 2.3.1.4 Biofilms in radionuclide bioremediation

Single or multiple microbial populations attached to abiotic or biotic surfaces through extracellular polymeric substances (EPS) are known as biofilms (Beyenal *et al.*, 2004). The researchers also reported that hexavalent U(VI) has been immobilised using biofilms of the sulphate-reducing bacterium *Desulfovibrio desulfuricans*.

Available evidence (Sarro *et al.*, 2005) suggests that accumulation of radionuclides, especially  $^{60}\text{Co}$  from the contaminated water, is caused by direct involvement of biofilm populations present in the spent nuclear fuel. Moreover, bioremediation can be accelerated by enhanced gene transfer methods among the biofilm microorganisms (Singh *et al.*, 2006) and facilitated by improving the chemotactic ability of microbial strains via genetic engineering approaches.

### 2.3.1.5 Siderophore-mediated radionuclide bioremediation

Iron exists in aerobic soils primarily as Fe(III), which is not readily water soluble and is unable to be acquired as a free ion by soil microbes. To avoid such difficulty, microorganisms produce siderophores, which are low-molecular-weight chelating agents that bind to iron and transport it into the cell through an energy-dependent process (Pierwola *et al.*, 2004). Renshaw and his colleagues (2003) also reported that siderophores (highly specific for Fe(III)) are able to bind effectively to actinides such as thorium, uranium, neptunium and plutonium.

When incubated with the siderophore desferrioxamine (DF), *microbacterium flavescens* has been observed to be bound to Pu(IV), Fe(III) and U(VI) (John *et al.*, 2001). Using transport proteins, the microbial cells were able to consume the Pu-siderophore complex at a much slower rate than they degrade the Fe-siderophore complex, while the U-siderophore complex was not degradable. The two complexes, Pu(IV)-DF and Fe(III)-

DF, mutually inhibit the uptake of one another, indicating that they may compete for the same binding sites or transport mechanisms within the microorganism. Also, the two complexes are said to be recognised by the same bacterial uptake because their structures (Pu(IV)-DF and Fe(III)-DF) are similar.

### 2.3.2 Microbial colonisation of radioactive environments

Studies involving microbial treatment of radioactive material are not new. Many studies have been performed in industries where the waste is treated with processes that remove, immobilize, or detoxify heavy metals and radionuclides before it is disposed. It has been known for centuries that micro-organisms possess a potential to reduce metals (Lloyd, 2003). In addition, certain microbes are able to exist in extreme conditions, including high radiation fields. Chicote et al (2004) characterized microorganisms found on the walls of a spent nuclear fuel storage pool at a Spanish nuclear power plant. They identified six bacteria via molecular 16S ribosomal RNA gene analysis. These bacteria were affiliated with  $\beta$ -Proteobacteria, *Actinomycetales* and the *Bacillus/Staphylococcus* groups. A fungus related to *Aspergillus* was also identified. Successful applications of these species include the microbial mediated precipitation of uranium from contaminated groundwater (Gorby and Lovley, 1992). In this process highly soluble hexavalent uranium [U(VI)] is enzymatically reduced with iron- and sulfate-reducing bacteria to tetravalent uranium [U(IV)], which precipitates as uraninite ( $UO_{2(s)}$ ) (Lovley and Phillips, 1992).

Numerous anaerobic denitrifiers, fermenters, sulphate reducers and methanogens were found isolated at the Severnyi repository for low-level liquid radioactive wastes in Eastern Siberia, Russia. These bacteria were cultured from water samples collected at depths ranging from 162 to 405 m below sea level (Nazina et al., 2004). Cell numbers and rates of a range of respiratory processes of species were higher in the zone of radioactive waste dispersion than in the background areas. One possible explanation for the co-location of bacteria and dispersed waste is that microbial gas production could

have resulted in repository pressure increase and subsequent discharge of wastes (Nazina et al., 2004).

Over decades of nuclear industry development, considerable quantities of natural and artificial radionuclides have been released to the environment. Major contributors to these releases have been, nuclear weapons testing, accidents involving radioactive material, and faulty storage of nuclear materials (Ruggiero et al., 2005). In many cases, storage of the nuclear waste has severely been compromised, which resulted in contamination of groundwater and soil. Chemical remediation techniques that are currently used are expensive and suffer from several technical limitations. New research is focused on the interaction of microorganisms with key radionuclides in developing cost-effective bioremediation approaches for decontamination of sediments and water impacted by nuclear waste (Lloyd et al., 2003). Passive *in situ* biological treatment processes that harness natural biogeochemical cycles for key radionuclides are highly desirable (Lloyd and Renshaw, 2005).

Methods for efficient separation of  $^{14}\text{C}$  from irradiated graphite are being studied by the PBMR Waste Minimization Project team. As the first phase of the research project, microbial treatment of waste graphite is being pursued. The project is currently at the proof-of-concept phase, with experiments being performed to systematically identify microbial species adaptable to the chemical and radiation environment of the system (Dunzik-Gougar et al., 2008).

## **2.4 Applicable theory of measurement methods**

### **2.4.1 Spectrophotometry**

Depending on the objectives of the study, bacteria can be grown using either solid or liquid media. Solid media are used to isolate bacterial strains, while the liquid medium (broth) can be used when measurements of growth are required (Johnston, 2007). The most common environmental conditions considered when growing bacteria are temperature, pH, oxygen, light, salt/sugar concentration and special nutrients. Each bacterium has an optimum range of these conditions within which it grows at a maximum rate (Prescott et al., 2005). Among other techniques, bacterial growth can be measured by monitoring pH and turbidity changes in liquid growth medium. Both properties are being measured in this research.

Spectrophotometry is used to measure the relative numbers of bacterial cells present in a liquid culture. The spectrophotometer sends visible light through a bacterial culture broth sample and records the amount of light that reaches a detector on the other side. The difference between the intensities of emitted and detected light is correlated to a turbidity value (absorbance). The greater the bacterial cell number, the higher the absorbance value. The most commonly used wavelengths for measurements of bacterial turbidity include 540nm, 600nm or 660nm (Madigan et al., 2003). Turbidity is the cloudiness that appears in broth culture as a result of an increase in bacterial cell numbers. A blank sample, consisting of a tube containing sterile media, is used to calibrate the instrument for a given set of measurements (Nester, 2004).

## 2.4.2 Liquid scintillation counting

### 2.4.2.1 Theory

Liquid Scintillation Counting (LSC) is a type of analysis, which has been used for many biological research applications and is a highly efficient method for the detection of low energy radiation such as  $^{14}\text{C}$  and  $^3\text{H}$ . One advantage of the LSC over other radiation detection systems is that the sample is placed within the detector, whereas in other systems the radioactive sample is brought only near the detector and the low energy radiation emitted from the sample must pass through a barrier (e.g. air or gaseous medium), often not reaching a detector. With liquid scintillation counting, the radiation has a better chance of being detected because it is within the detector (Kessler, 1989).

The liquid scintillation technique is based on transferring the energy of radioactive decay to a molecule that "scintillates", or gives off visible light, on absorption of that energy. As shown in Figure 2.1, in the LSC instrument, a sample is contained in a vial together with a scintillant solution. The radioisotope,  $^{14}\text{C}$ , undergoes radioactive decay, releasing a  $\beta$ -particle. The kinetic energy of the particle radiation is absorbed by the solvent molecule, resulting in that molecule being in an excited state (electrons are in a higher than usual energy level) (McDowell and McDowell, 1994). The primary solute molecule absorbs energy from the excited solvent molecules, and then re-emits energy in the form of light at a wavelength compatible with the response of a photomultiplier (Kessler, 1989).

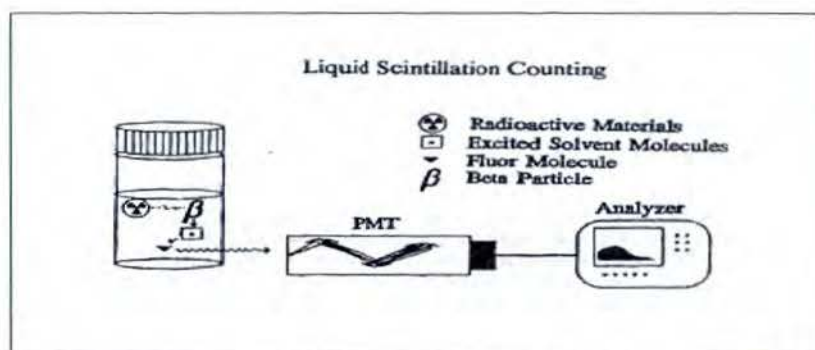


Figure 2.1: Liquid scintillation counting (Kessler, 1989)

The photosensitive device amplifies the emitted light from the sample vial, and the amplified signal is then converted to pulses of electrical energy which are registered as counts. The accumulated counts are separated in channels, with the amplitude determining the energy channels. The LSC data analysis software then performs correction calculations to convert the counts per minute (CPM) to disintegrations per minute (DPM) (Kessler, 1989).

### 2.4.2.2 Practice

There are several counting interferences that come with the use of LSC instruments. The interferences include quenching, chemiluminescence and heterogeneous samples (Kessler, 1989). Quenching is any factor which reduces the energy transfer efficiency or interference with the production (transmission) of light from the radioactive sample. There exist two types of quench as shown in Figure 2.2, i.e., chemical and colour quench (Gibson, 1980). Chemical quench results during the transfer of energy from the solvent to the scintillant, while colour quench occurs from attenuation of the photons produced by colour in the solution (Birks, 1971).

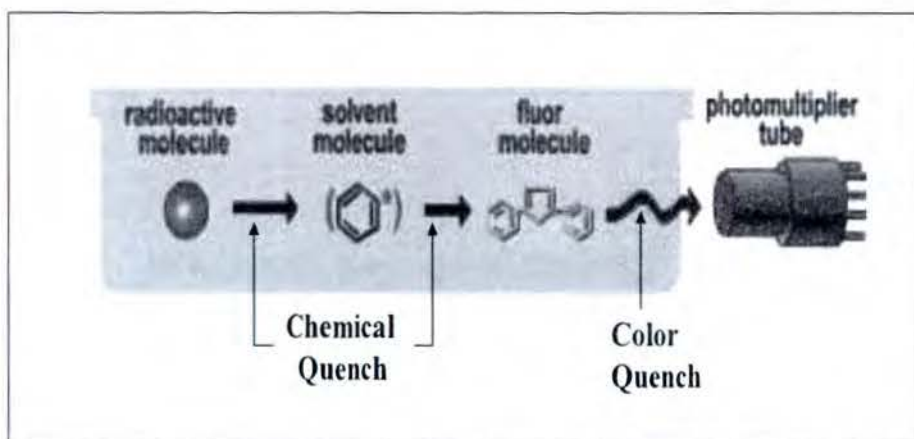


Figure 2.2: Quenching in the energy transfer process (Birks, 1971)

Quench is measured via two methods of spectral analysis. The first method is the Spectral Index of the Sample (SIS), which uses the sample isotope spectrum to monitor the quench of the solution. The second method used is the transformed Spectral Index of the External Standard (t-SIE), which is calculated from the Compton spectrum induced in the scintillation cocktail by an external source. The t-SIE value, scaled from 0 (most quenched) to 1000 (unquenched), is determined from a mathematical transformation of the generated spectrum (Thompson, 2001).

In his report, Thompson (2001) suggests preparation of standard quench curves as a way to correct quenching. The standard quench curves are generated from the analysed series of standard samples, prepared with the absolute radioactivity (DPM) per vial kept constant and the amount of quench increased from vial to vial. When a quench curve is generated, the DPM value in each analysed standard sample is known. The CPM values of each standard sample are measured when the (standard) samples are analysed. A correlation is drawn amongst the determined quench curves, the DPM and the measured CPM values. From this, counting efficiency is determined (as in equation 1) and stored in the instrument computer. The counting efficiency is then used to correct the CPM of an unknown sample to DPM.

$$\frac{\text{CPM}}{\text{DPM}} \times 100 = \% \text{ counting efficiency} \quad (1)$$

In the LSC instrument, optimum counting performance is achieved when the analysed sample is in a homogeneous phase (in solution) with the scintillation cocktail. Radionuclides not in the organic phase (i.e. precipitated, adsorbed, or in the separate liquid phase) yield lower counting efficiency than the potential counting efficiency of the cocktail. To correct this, aqueous-based solubilizer solutions are added to the organic samples to break down the macro-molecular structures of their bacterial cells (Kessler, 1989).

## 3. EXPERIMENTS

The experiments described in this thesis were designed and performed to establish baseline condition for future experiments using irradiated graphite. Experimental objectives were to (1) test the compatibility of candidate bacteria with a graphite system, (2) identify the bacterial growth medium optimum for the system, and (3) create a bioreactor environment in which bacteria consume a source of surrogate graphite  $^{14}\text{C}$ .

### 3.1 Bacterial growth in the presence of graphite

Research objectives (1) and (2), above, were achieved through a series of experiments resulting in the selection of bacteria species and medium composition range to be used for continuing experiments. Process details of these scoping experiments are presented here.

#### 3.1.1 Bacterial inoculum preparation

A variety of bacteria were tested for compatibility with the experimental system. Five bacterial species (*Bacillus subtilis*, *Bacillus megaterium*, *Pseudomonas putida*, *Pseudomonas fluorescence* and *Enterococcus*) were supplied by the Department of Microbiology and Plant Pathology at the University of Pretoria. These species were chosen because they are environmental strains and have dormant stages (alive but have slower metabolism), which makes them survive in harsh conditions. Each species was separately inoculated onto Nutrient Agar plates and maintained at 28°C for 48 hours. The resulting agar plates, containing single bacterial isolates, were then stored at 4°C for later use. In addition to these single species, a freeze-dried mixed bacterial culture was provided by PBMR (Pty) Ltd. The identity of these species is considered proprietary and was not provided to the researchers.

To grow sufficient quantities of bacteria for planned experiments, each type was prepared by inoculating 250 ml of Nutrient broth in 500 ml Erlenmeyer flasks. Following inoculation, flasks were incubated in a shaking incubator at 28°C for a 48-hour period.

### 3.1.1.1 Test tube reactors

Microbiologists consulting on this project noted the relatively inhospitable environment of graphite. There was concern that bacteria would not grow on and/or around this material, which is a very nonreactive, inorganic. As such, experiments were designed to determine if graphite had any significant effect on bacterial growth.

Bacteria were contacted with solutions containing different ratios of carbon sources glucose and graphite. Štyriaková enrichment medium (as described in Table 3.1) provided not only the medium for bacterial growth, but also for replacing some portion of the standard carbon source, glucose, with graphite powder.

Table 3.1: Composition of Štyriaková medium (Štyriaková, 2004)

Component	Mass (g/L of distilled water)	Molarity (mol/L)	Molarity normalized with respect to carbon (in glucose)
NaH <sub>2</sub> PO <sub>4</sub>	0.42	0.0035	0.0058
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.80	0.0061	0.010
NaCl	0.19	0.0033	0.0054
Glucose (C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> ·H <sub>2</sub> O)	19.80	0.0999	0.17
Carbon in glucose (C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> )	7.2	0.60	1

Glucose and graphite combinations were tested in triplicate in small-scale test tube “bioreactors”. To facilitate preparation of the different medium compositions, a starting solution containing only sodium biphosphate (NaH<sub>2</sub>PO<sub>4</sub>), ammonium sulfate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), and sodium chloride (NaCl) was prepared. Each test tube started with 10 ml of this solution, followed by glucose and/or graphite powder according to the test plan in Table 3.2.

Table 3.2: Bioreactor compositions for graphite effects experiments

Composition variation	Glucose		Graphite		Glucose C: Graphite C
	Mass (g)	Mole C	Mass (g)	Mole C	
1	20	0.61	0	0	1:0
2	15	1.10	5	0.42	1:0.92
3	10	0.30	10	0.83	1:2.8
4	5	0.15	15	1.25	1:8.3

To prepare bacteria for these bioreactors, the cultures were isolated from nutrient broth via centrifuge and the resulting pellets suspended in distilled water. From these suspensions, 100  $\mu$ L of the appropriate species was added to each test tube. The resulting test-tube bioreactors (72 of them) were incubated and shaken for 288 hours at 28°C.

The pH of each bioreactor liquid phase was measured every 72 hours throughout the 288 hour period. Change in pH is an indirect indication of bacterial growth, given that bacteria produce metabolic acids, which lower pH of the aqueous medium.

### 3.1.1.2 Flask reactors

Results of the test-tube scale experiments were inconclusive. Even with no glucose replacement, bacterial growth was less than expected. One possible explanation for the lack of growth is lack of required oxygen due to reactor geometry. The aerobic bacteria had limited opportunity for oxygen exposure due to the relatively small surface area of the reaction medium.

To test this theory, a larger-scale, simplified variation of the test tube experiments was performed in triplicate in 500-ml Erlenmeyer flasks chosen to increase the oxygenation of the system. Flasks were prepared with 250 ml of the starting solution and a small piece of graphite (~ 1 cm<sup>3</sup>), instead of graphite powder. Only a subset of the many glucose, graphite and bacteria combinations was performed on this scale. *Bacillus megaterium*, *pseudomonas fluorescence* and *Enterococcus* species were eliminated as marginal

candidates and only *Bacillus subtilis*, *Pseudomonas putida* and mixed culture inoculates (1 ml) were individually added to two of eight flasks, to total 6 flasks. It was assumed that the number of cells in each bioreactor was the same because the same volume of inoculate solution was used. To one of each flask pair containing a given inoculate, glucose was added in the quantity required to create the standard Štyriaková medium. No glucose was added to the other member of the flask pair. The two remaining flasks served as control reactors, each containing graphite, one containing glucose and neither containing bacteria, as described in Table 3.3.

Table 3.3: Bioreactor configuration for flask-scale, graphite effects experiments

Flask reactor	Graphite piece (~1cm <sup>3</sup> )	Glucose (0.1 moles/L medium)	Bacteria species
1	X		<i>Bacillus subtilis</i>
2	X	X	<i>Bacillus subtilis</i>
3	X		<i>Pseudomonas putida</i>
4	X	X	<i>Pseudomonas putida</i>
5	X		Mixed culture
6	X	X	Mixed culture
7	X		None
8	X	X	None

## 3.2 Introduction of carbon-containing salts

The third objective of the experiments was to create a bioreactor environment in which bacteria consume a source of surrogate  $^{14}\text{C}$  representing  $^{14}\text{C}$  in irradiated graphite. Choice of a chemical surrogate for  $^{14}\text{C}$  in graphite was difficult, because little is known of its chemical bonding. As previously discussed, there is evidence suggesting  $^{14}\text{C}$  bonding with some combination of N, O, H and C (Marsden et al., 2002). Bonding with these elements is consistent with the results of studies indicating the location of  $^{14}\text{C}$  in the irradiated graphite outer surface, where open pores may contain water and nitrogen impurities from air exposure.

Two salts with carbon-containing anions ( $\text{NaCH}_3\text{CO}_2$  and  $\text{NaHCO}_3$ ) were chosen to represent  $^{14}\text{C}$  in these experiments. The salts were chosen because they have carbon bonding in the acetate and carbonate ions that may be representative of the bonding of  $^{14}\text{C}$  on the graphite surface after irradiation. In the acetate and bicarbonate ions, there are carbon-oxygen single and double bonds and carbon-hydrogen bonds. Anions containing carbon-nitrogen bonds, nominally cyanides, were considered but deemed too problematic to warrant use at this early stage of the investigation. Sodium was the cation of choice because it is a necessary component of microbial reproduction.

### 3.2.1 Sodium replacement

Because sodium is a standard, and necessary, component of the Štyriaková medium used in these experiments, it was decided to compare bacterial growth in a system containing the usual sodium source ( $\text{NaCl}$ ) with growth in medium containing alternate sources of sodium, the carbon-containing salts. Sodium is required for bacterial growth and replacing it with the aforementioned salts avoids having it in excess in the medium.

During these experiments, Štyriaková medium without NaCl was prepared according to Table 3.1. 250 ml of the prepared medium was added to each 500 ml Erlenmeyer flask along with one of three salts ( $\text{NaCH}_3\text{CO}_2$ ,  $\text{NaHCO}_3$  and  $\text{NaCl}$ ). Four reactor sets correspond to 4 salt masses (Table 3.4), chosen according to the mass of glucose being replaced. Reactors in set 1 contained 2 g of single salt. Reactors in sets 2 – 4 contained 3g, 4g, and 5g of salt, respectively. Three bioreactors were prepared for each composition to allow for better statistics in the resulting data. Each bioreactor was then inoculated with 200  $\mu\text{l}$  of the bacterial mixed culture inoculum. The reactors were incubated for 72 hours in a shake incubator at 28°C. pH and absorbency (at 600 nm) of the liquid medium were measured daily to monitor bacterial growth.

Table 3.4: Bioreactor salt content for sodium replacement experiments

Sodium salt	Reactor set 1 (2 g salt)	Reactor set 2 (3 g salt)	Reactor set 3 (4 g salt)	Reactor set 4 (5 g salt)
	Molar Concentration of Sodium Salt in the liquid medium			
$\text{NaCH}_3\text{CO}_2$	0.097	0.145	0.193	0.241
$\text{NaHCO}_3$	0.094	0.141	0.189	0.236
$\text{NaCl}$ (Control)	0.136	0.203	0.271	0.339

### 3.2.2 Glucose replacements

The standard source of carbon in Štyriaková enrichment medium is glucose. In order to encourage consumption of an alternative carbon source, bioreactors were operated with some part of usual glucose content replaced by one or both of the carbon-containing salts. Glucose was replaced by the carbon-containing salts according to Table 3.5. Each bioreactor (500-ml Erlenmeyer flask with 250 ml of aqueous medium) was inoculated with 200  $\mu\text{l}$  of the bacterial mixed culture inoculum. The reactors were prepared in triplicate and incubated in a shake incubator at a temperature of 28°C for a period of 96 hours. Daily measurements of pH and absorbency (at 600 nm) were performed to monitor bacterial growth.

Table 3.5: Bioreactor carbon (and NaCl) content for glucose replacement experiments

Bioreactor	Carbon concentration (moles/L medium)			Na concentration (moles/L medium)
	NaCH <sub>3</sub> CO <sub>2</sub>	NaHCO <sub>3</sub>	Glucose	NaCl
Reactor 1	0.48	0	0	0.00
Reactor 2	0	0.24	0	0.00
Reactor 3	0.24	0.12	0	0.00
Reactor 4	0	0	0.60	0.0033
Reactor 5	0	0	0.00	0.0033

### 3.2.3 Sodium and glucose replacements

To assess bacterial growth in the presence of alternative sources of sodium and carbon together, flask bioreactors were prepared with 250 ml of the medium (Štyriaková without glucose and sodium chloride) as described in Table 3.6 to Table 3.8. The quantities of the carbon-containing salts were chosen based on mass of glucose in the medium. Each 500-ml Erlenmeyer flask was inoculated with 200 µl of the bacterial mixed culture inoculum. No sodium chloride was added to the reactors because the carbon-containing salts provided the necessary sodium. Each reactor configuration was prepared in triplicate and incubated for 120 hours in a shake incubator at 28°C. The results of pH and absorbency (at 600 nm) measurements were used to monitor bacterial growth.

Table 3.6: Glucose, NaCH<sub>3</sub>CO<sub>2</sub> and NaHCO<sub>3</sub> concentrations in the 100% glucose bioreactor system for carbon and sodium replacement experiments

Bioreactor (Acetate C : Bicarbonate C : Glucose C)	Concentrations					
	NaCH <sub>3</sub> CO <sub>2</sub>		NaHCO <sub>3</sub>		Glucose	
	g/L	mol C/L	g/L	mol C/L	g/L	mol C/L
Reactor 1 (1:0.12:4)	6.19	0.1509	1.58	0.0188	19.80	0.5997
Reactor 2 (1:0.21:4.5)	5.42	0.1322	2.38	0.0283	19.80	0.5997
Reactor 3 (1:0.5:6.4)	3.87	0.0944	3.96	0.0472	19.80	0.5997
Reactor 4 (1:1.2:11)	2.32	0.0566	5.54	0.0660	19.80	0.5997
Reactor 5 (1:2:16)	1.55	0.0378	6.37	0.0759	19.80	0.5997

Table 3.7: Glucose, NaCH<sub>3</sub>CO<sub>2</sub> and NaHCO<sub>3</sub> concentrations in the 50% glucose bioreactor system carbon and sodium replacement experiments

Bioreactor (Acetate C : Bicarbonate C : Glucose C)	Concentrations					
	NaCH <sub>3</sub> CO <sub>2</sub>		NaHCO <sub>3</sub>		Glucose	
	g/L	mol C/L	g/L	mol C/L	g/L	mol C/L
Reactor 1 (1:0.12:2)	6.19	0.1509	1.58	0.0188	9.90	0.2998
Reactor 2 (1:0.21:2.3)	5.42	0.1322	2.38	0.0283	9.90	0.2998
Reactor 3 (1:0.5:3.2)	3.87	0.0944	3.96	0.0472	9.90	0.2998
Reactor 4 (1:1.2:5.3)	2.32	0.0566	5.54	0.0660	9.90	0.2998
Reactor 5 (1:2:8)	1.55	0.0378	6.37	0.0759	9.90	0.2998

Table 3.8: Glucose, NaCH<sub>3</sub>CO<sub>2</sub> and NaHCO<sub>3</sub> concentrations in the 20% glucose bioreactor system carbon and sodium replacement experiments

Bioreactor (Acetate C : Bicarbonate C : Glucose C)	Concentrations					
	NaCH <sub>3</sub> CO <sub>2</sub>		NaHCO <sub>3</sub>		Glucose	
	g/L	mol C/L	g/L	mol C/L	g/L	mol C/L
Reactor 1 (1:0.12:0.8)	6.19	0.1509	1.58	0.0188	3.96	0.1199
Reactor 2 (1:0.21:1)	5.42	0.1322	2.38	0.0283	3.96	0.1199
Reactor 3 (1:0.5:1.3)	3.87	0.0944	3.96	0.0472	3.96	0.1199
Reactor 4 (1:1.2:2.1)	2.32	0.0566	5.54	0.0660	3.96	0.1199
Reactor 5 (1:2:3.2)	1.55	0.0378	6.37	0.0759	3.96	0.1199

### 3.3 Introduction of carbon-14 containing salts

Experiments with the non-active carbon-containing salts allowed assessment of any significant effects of the salts on bacterial growth. Results of these experiments may indicate if the salts did NOT allow growth; however, the presence of growth was not necessarily an indication that the salts SUPPORTED growth. In other words, there was no direct evidence of having reached the third objective: to create a bioreactor environment in which bacteria consume a source of surrogate <sup>14</sup>C graphite.

To gather such evidence, one must be able to distinguish between the carbons in the glucose and those in the salts. The distinction was possible with the introduction of <sup>14</sup>C-labelled NaC<sub>2</sub>H<sub>3</sub>O<sub>2</sub> and NaHCO<sub>3</sub>. Tracking the location of <sup>14</sup>C before, during and after reactor operation would provide information regarding the type of processes affected on the salts. The total <sup>14</sup>C inventory begins in the liquid medium, where it is provided as an alternative (to glucose) food source for the microbes. As the reactor operates, a shift of <sup>14</sup>C content from liquid to solid phase (bacterial biomass) and/or gaseous phase (bioreactor off-gas) may indicate microbial processing of the <sup>14</sup>C salt.

### 3.3.1 Experimental set-up

Figure 3.1 illustrates the configuration of the experimental apparatus. 500-ml Erlenmeyer flasks containing the growth medium,  $^{14}\text{C}$ -labelled salt and the microbial culture inoculum were used as bioreactors. Because it was crucial to account for all  $^{14}\text{C}$  in the system, the apparatus was designed to oxidise the off-gas ( $\text{CH}_4$ ,  $\text{CO}$  and  $\text{CO}_2$ ) produced by bacteria during experiments to  $\text{CO}_2$  (and  $\text{H}_2\text{O}$ ). Regulated carbon-free-air carried the off-gas to a tube furnace, where it was oxidized, and the resulting  $\text{CO}_2$  was trapped in a basic (1M NaOH) solution.

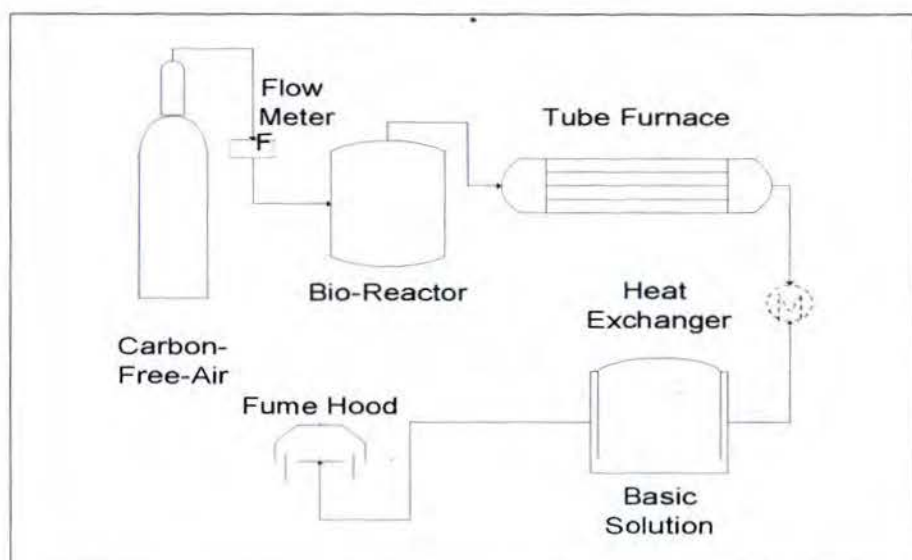


Figure 3.1: Schematic layout of the experimental set-up

### 3.3.2 Experiments with $^{14}\text{C}$ -labelled sodium acetate

During these experiments 250 ml Štyriaková growth medium was prepared as in Table 3.1, without NaCl and with (0.12 M) glucose. NaCl was replaced (in the medium) with  $^{14}\text{C}$ -labelled sodium acetate to avoid having sodium in excess. The quantity of glucose was chosen based on previous experiments (section 3.2.3). Also added was 1.7 ml of the  $^{14}\text{C}$ -labelled  $\text{NaCH}_3\text{CO}_2$  (GE healthcare UK limited, Buckinghamshire, UK) solution at an activity concentration of 62.5 Bq per 250 ml bioreactor. The quantity of acetate solution used in the experiments was chosen to accommodate the detection sensitivity of the liquid scintillation counting instrument. It was determined that a minimum activity of 250 Bq per

1L bioreactor would allow statistically significant counting of the activity in each reactor phase after operation. The bioreactors (prepared in triplicate) were inoculated with 1 ml of the mixed culture inoculum and incubated at an ambient temperature of 25°C. Carbon-free-air (10 L.hr<sup>-1</sup>) carried the off-gas from the bioreactor through a tube furnace operated at a temperature of 800 °C. The oxidized gas was then trapped in 500 ml NaOH (1M) base solution. The reactors were operated and stopped for sample analysis as shown in Table 3.9. The times were chosen based on experiments performed in the design case study (in sections 3.2.2 and 3.2.3), where reactors were operated from 24 to 120 hours.

Table 3.9: Bioreactor operation times

Reactor	Run time (hr)
1	24
2	48
3	72
4	96
5	120

### 3.3.3 Experiments with <sup>14</sup>C-labelled sodium bicarbonate

These experiments were conducted as described in section 3.3.2, with the exception that the salt used was <sup>14</sup>C-labelled NaHCO<sub>3</sub>, also from a stock solution with activity concentration of 62.5 Bq per 250 ml bioreactor (GE healthcare UK limited, Buckinghamshire, UK). The bioreactors were operated from 24 to 72 hours and then stopped for sample analysis. The longer operation times (96 and 120 hours) were eliminated based on observations made during the experiments with Na<sup>14</sup>CH<sub>3</sub>CO<sub>2</sub>. After 72 hours of reactor operation, it was decided to not continue the experiments any further as there was little change in bacterial growth rate.

“Control” reactors were operated with  $^{14}\text{C}$ -labelled sodium acetate or bicarbonate, but no bacteria. Results from these experiments allowed assessment of the radioactivity distribution through the different phases (solid, liquid and gas) from processes other than bacterial action.

### **3.3.4 Liquid scintillation counting sample preparation**

Samples from all three bioreactor phases (solid, liquid and gas) were analysed for  $^{14}\text{C}$  content via liquid scintillation counting with a Tri-Carb 2900TR liquid Scintillation Analyzer (PerkinElmer). Liquid and solid samples from the reactor required separation and treatment prior to mixing with the appropriate scintillant cocktail. The liquid media from the bioreactors were transferred into centrifuge bottles and centrifuged for 20 min at a speed of 5000 rpm to separate the liquid medium from the bacterial biomass. The resulting supernatant was pipetted away from the biomass pellet into an empty container. From this container, three liquid medium samples (2 ml each) were separately added to three LSC vials containing 10 ml of UltimaGold® high flashpoint LSC-cocktail (PerkinElmer).

To break down the macro-molecular structures of the bacterial cell biomass pellet, 10 ml of Solvable™ Aqueous-based Solubilizer (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) were added to each centrifuge bottle. The pellet-solvent mixtures were incubated at 25°C for 1 hour. Three samples (2 ml each) of the dissolved bacterial suspensions were separately added to three LSC vials containing 10 ml of UltimaGold® high flashpoint LSC-cocktail (PerkinElmer).

$^{14}\text{C}$  content in the gas phase was determined via analysis of the NaOH solutions, which collected the  $\text{CO}_2$  produced from reactor off-gas oxidation.

### 3.3.5 Liquid scintillation quenching studies

Quenching is a reduction in the LSC's system efficiency resulting in energy loss. Many counting interferences occur as a result of colour and optical quenching, also due to chemiluminescence and photoluminescence. The degree of quenching, nature of the sample, and cocktail used affects the efficiency, which in turn affects the results of sample analysis. In correcting the interference, compatibility between the sample and scintillant solution is a key factor in minimizing counting interference. The ideal sample-scintillant mixture is clear and colourless. However, chemical incompatibilities between sample and scintillant can produce cloudy or coloured mixtures, which do not perform well for LSC application.

Experiments were performed to determine (1) which of the scintillant solutions (UltimaGold or Hionic-Fluor) would be more compatible with the bioreactor aqueous medium, and (2) which combination of scintillant and basic solution (sodium hydroxide (NaOH) and potassium hydroxide (KOH)) would be most compatible with LSC analysis.

#### 3.3.5.1 Aqueous phase sample-scintillant compatibility tests

For study objective (1), sample-scintillant compatibility was tested using each of the two scintillants, UltimaGold and Hionic-Fluor. The scintillants were chosen because they are compatible with alkaline samples.

In preparing for the quench tests, 10 ml of UltimaGold and Hionic-Fluor cocktail were each added to 10 vials (five vials for each cocktail). Each appropriate vial was added with  $^{14}\text{C}$ -labelled  $\text{NaCH}_3\text{CO}_2$  (GE healthcare UK limited, Buckinghamshire, UK) solution at an activity concentration of  $148 \text{ Bq}\cdot\text{ml}^{-1}$  as illustrated in Table 3.10. Compatibility was checked by keeping the scintillant volume in the vial constant, while varying the sample volumes. The vials were counted for 1 hour via the LSC. Only the  $^{14}\text{C}$ -labelled  $\text{NaCH}_3\text{CO}_2$  salt was used, as it was thought that the bicarbonate was more likely to decompose to  $\text{CO}_2$ , thereby negatively affecting the LSC analysis.

Table 3.10: Volumes of  $^{14}\text{C}$ -labelled  $\text{NaCH}_3\text{CO}_2$  in vials containing UltimaGold and Hionic-Fluor

Cocktail	Sodium acetate volume (ml per vial)			
	Trial 1	Trial 2	Trial 3	Trial 4
UltimaGold	2.0	3.0	5.0	7.0
Hionic-Fluor	2.0	3.0	5.0	7.0

Results of liquid scintillation counting of samples were as expected with respect to a decrease in activity (counts) when the sample volume (in a vial) was increased. The relatively consistent difference in counted activity (reference Figure 3.2) between the two (UltimaGold and Hionic-Fluor) solutions suggests that the samples are more compatible with UltimaGold over Hionic-Fluor.

As a result of these tests, UltimaGold was chosen for use with the reactor liquid medium samples obtained in the radioactive experiments.

### 3.3.5.2 Hydroxide solution-scintillant compatibility tests

Presented in this section for objective (2) are study experiments performed to determine which of the basic solutions (NaOH or KOH) should be used for adequate  $\text{CO}_2$  absorption, while not adversely affecting LSC counting. 10 ml of UltimaGold or Hionic-Fluor cocktail was added to a total of 10 LSC vials (five vials for each cocktail). Appropriate concentrations of the base solutions were added to each vial (Table 3.11 and Table 3.12). Concentrations of the basic solutions were varied to determine the quenching effect, if any. In particular, there was concern about the use of KOH due to the naturally occurring radioisotope  $^{40}\text{K}$  possibly causing counting interference. The vials were counted in the LSC for 1 hour.

Table 3.11: Concentration of NaOH in vial containing UltimaGold and Hionic-Fluor

Vial #	UltimaGold volume (ml)	Hionic-Fluor volume (ml)	NaOH concentration (Molar)
1	10.0	-	0.1
2	10.0	-	0.5
3	10.0	-	1.0
4	10.0	-	1.5
5	10.0	-	2.0
6	-	10.0	0.1
7	-	10.0	0.5
8	-	10.0	1.0
9	-	10.0	1.5
10	-	10.0	2.0

Table 3.12: Concentration of KOH in vial containing UltimaGold and Hionic-Fluor

Vial #	UltimaGold volume (ml)	Hionic-Fluor volume (ml)	KOH concentration (Molar)
1	10.0	-	0.1
2	10.0	-	0.5
3	10.0	-	1.0
4	10.0	-	1.5
5	10.0	-	2.0
6	-	10.0	0.1
7	-	10.0	0.5
8	-	10.0	1.0
9	-	10.0	1.5
10	-	10.0	2.0

The lowest counts for NaOH and KOH (Figure 3.2 and Figure 3.3), were attained when 1M and higher concentration basic solutions were separately added to vials containing 10 ml UltimaGold cocktail. UltimaGold cocktail has a dramatic quenching effect on both basic solutions above 0.5M concentration. Hionic-Fluor has minimal effect on the NaOH solutions but has a significant effect on activity counts for KOH solutions at higher concentrations. This difference between bases may be due to the presence of radioactive  $^{40}\text{K}$  in natural potassium compounds.

Based on these results, NaOH was used for  $\text{CO}_2$  collection and the resulting basic solutions were mixed with Hionic-Fluor for LSC analysis:

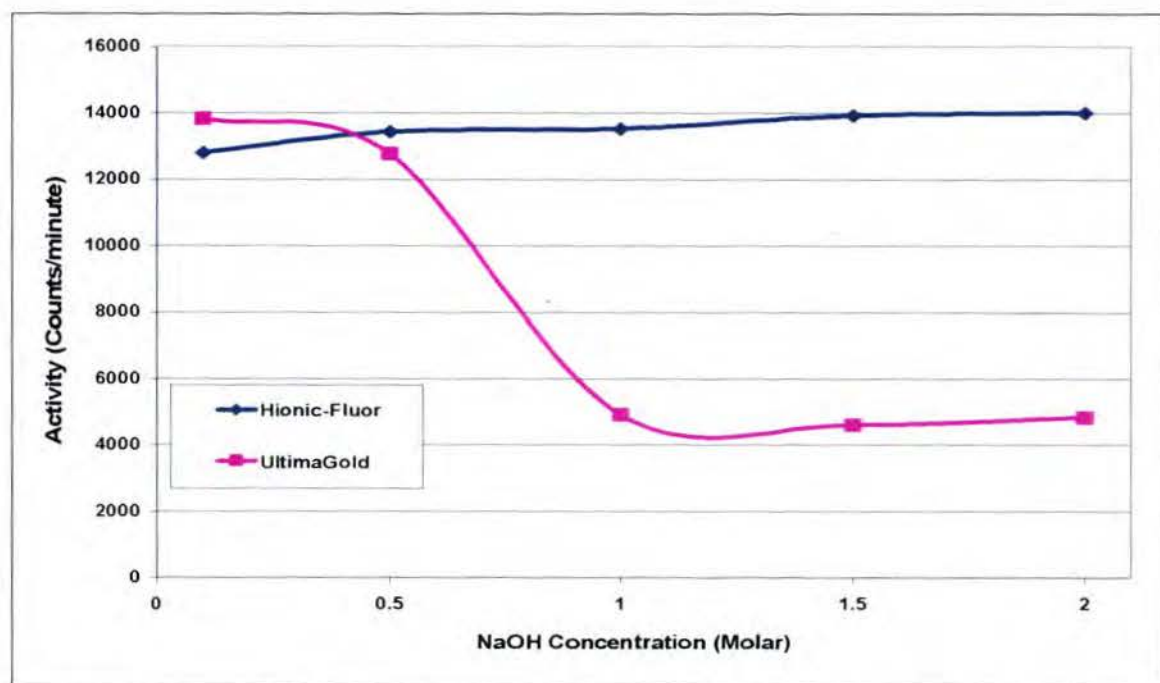


Figure 3.2: Sodium hydroxide sample-scintillant compatibility test graph

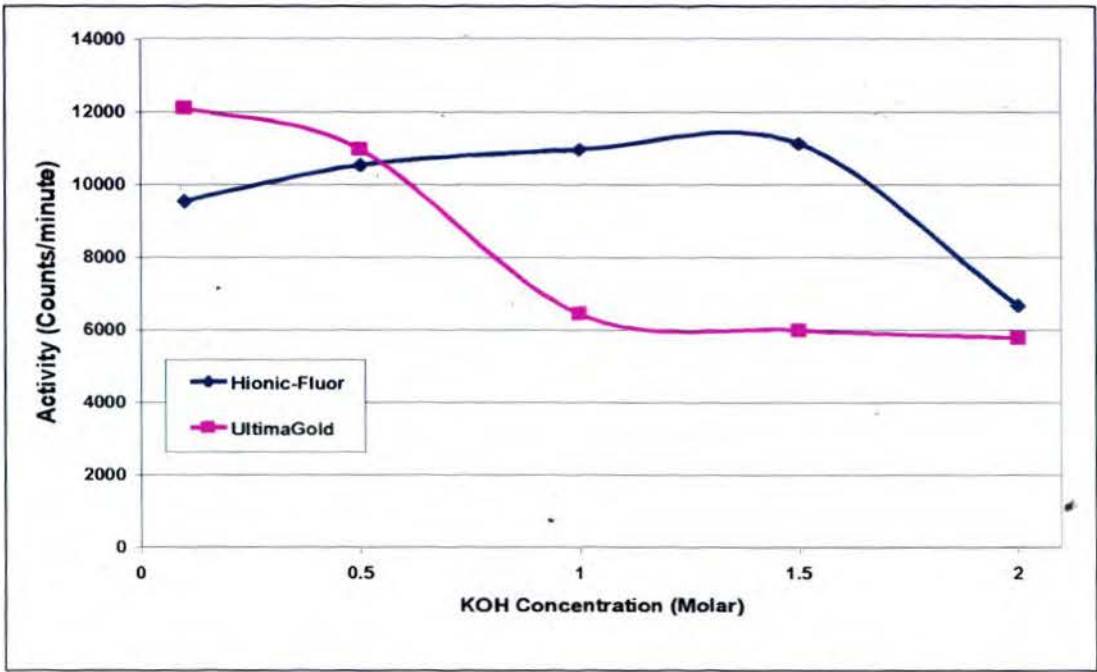


Figure 3.3: Potassium hydroxide sample-scintillant compatibility test graph

# 4. RESULTS AND DISCUSSION

In this chapter, the observations made and measurements taken during experimental work are presented and discussed. The experimental results are evaluated with respect to the objective for which the experiments were designed.

## 4.1 Bacterial growth in the presence of graphite

### 4.1.1 Test tube reactors

Figure 4.1 to Figure 4.7 are plots of pH data for bioreactors containing various glucose-to-graphite mass ratios. One of five single bacteria species or a mixed culture was introduced to each mass ratio system. The pH was used as the bacterial growth indicator for these experiments. As they multiply, bacteria produce metabolic acids that result in a drop in pH of the growth medium. Turbidity could not be measured, because the growth media in the test tube bioreactors was not of sufficient volume to be analysed via spectrophotometry.

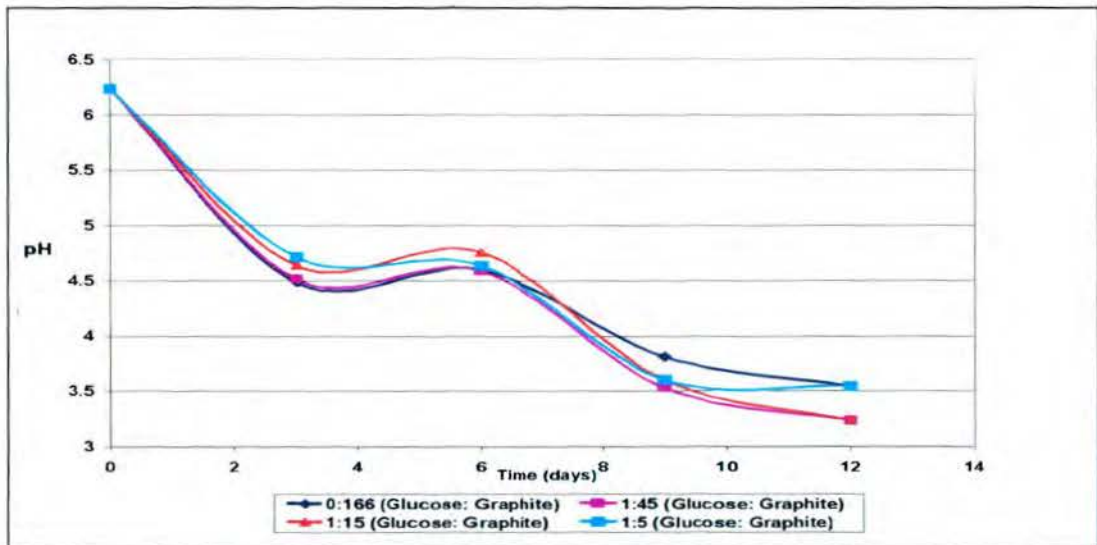


Figure 4.1: Growth curve for *Bacillus subtilis* species in the presence of glucose and/or graphite carbon sources

Figure 4.1 shows the pH trends for the four reactors containing species *Bacillus subtilis*. While this plot does allow comparison of trends over time, it is not useful for comparing amount of growth in each system as a function of the carbon source. The four bioreactors represented in the figure did not contain the same number of carbon atoms and, therefore, growth (and the associated pH change) occurred with different amounts of food. Ideally, the only variable in each system would be the chemical form of carbon. However, both chemical form and total quantity of carbon were changed for each reactor containing the same bacterial species.

The most meaningful manner in which to compare the growth (pH) data is with respect to atoms (or mole equivalents) of carbon in the system. Figure 4.2 provides a comparison of the cumulative change in pH per mole carbon as a function of time in the *Bacillus subtilis* reactors.

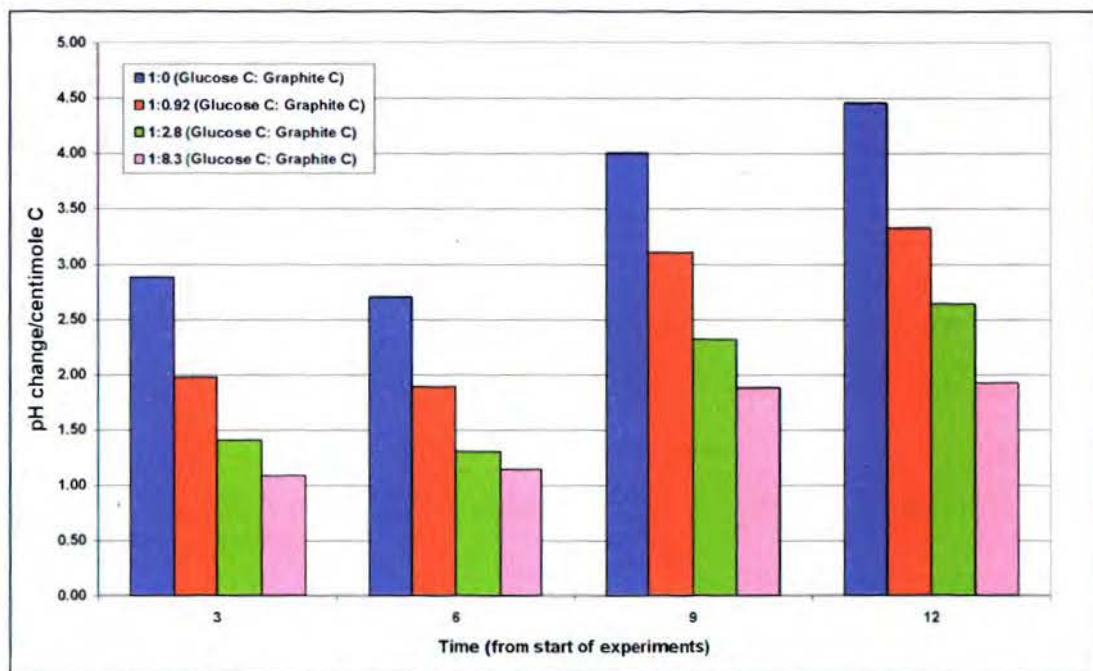


Figure 4.2: Cumulative pH change per centimole carbon as a function of time in the *Bacillus subtilis* reactors.

The most significant pH change per centimole of carbon (4.46 at 12 days) takes place in the reactor system with glucose and no graphite. This result is consistent with the ease with which bacteria are known to metabolize glucose. As the source of carbon changes from glucose to graphite, there is significantly less change in pH for each carbon (~ 1.9 at 12 days for the reactor system with a 1:8.3 glucose to graphite carbon ratio.) This trend likely is explained by the chemically inert nature of graphite. While it is possible that some of the graphite was metabolized by the bacteria, the trend of decreasing pH change indicates that less growth occurred per carbon when the carbon was in graphite instead of glucose.

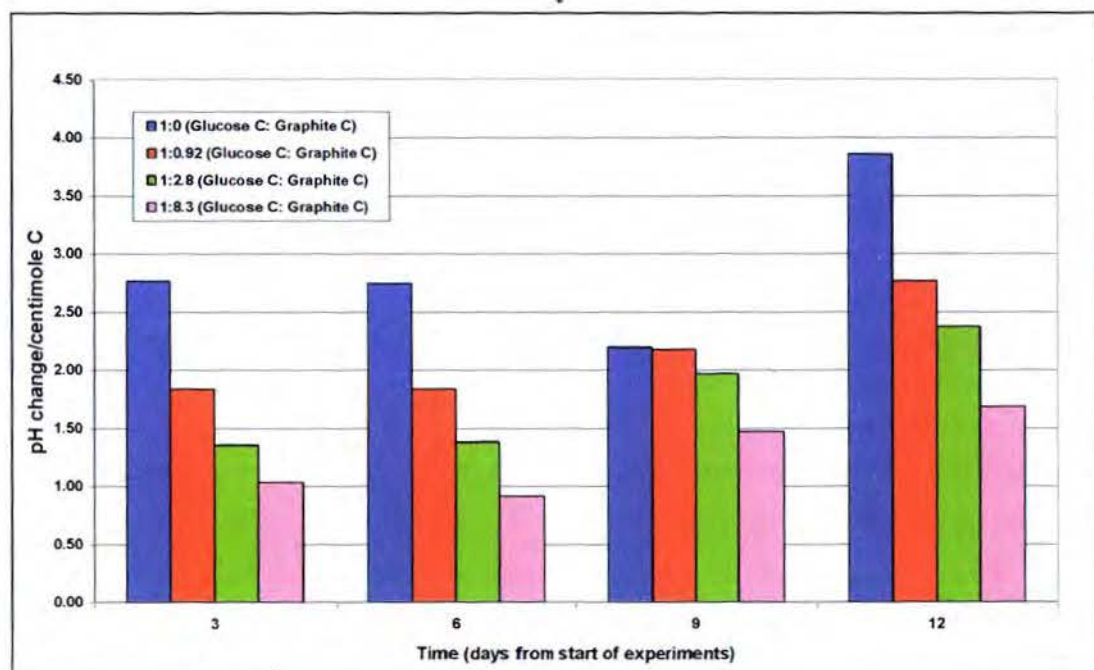


Figure 4.3: Cumulative pH change per centimole carbon as a function of time in the *Bacillus megaterium* reactors.

As with the *Bacillus subtilis*, the most significant pH change per centimole of carbon in the *Bacillus megaterium* reactors (Figure 4.3), takes place in the reactor system with glucose and no graphite. The pH change per centimole C is stable in the first 6 days, then drops (from ~2.7 to 2.2 by day 9) due to the depletion of glucose as a food source, and increases (by day 12), after the microbes start feeding on dead comrades. For reactors containing graphite, pH change was constant through 6 days and increased by small

increments for measurements taken at days 9 and 12. As would be expected, the graphite reactors with more glucose had larger pH changes.

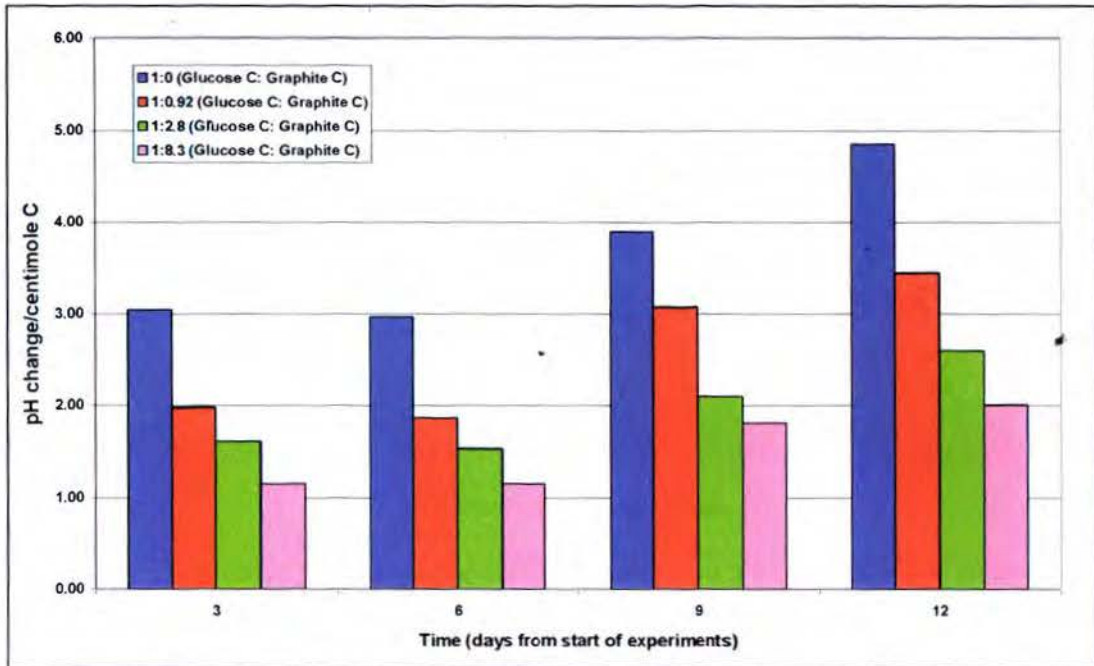


Figure 4.4: Cumulative pH change per centimole carbon as a function of time in the *Pseudomonas putida* reactors.

The pH change per centimole of carbon for *Pseudomonas putida* (Figure 4.4) started off steadily (~3.0 and ~2.9, respectively, at 3 and 6 days) for reactor systems with the glucose to graphite ratio of 1:0. The trend decreases with the change of carbon source from glucose to graphite. The graphite reactors' pH changes trended similarly to the *Bacillus megaterium* reactors (Fi. 4.3); though larger changes occurred in the *Pseudomonas putida* reactors.

The *Pseudomonas fluorescens* (Figure 4.5) pH change per centimole carbon data generally follows the same trend as that of the *Bacillus* reactors (Figure 4.2 and Figure 4.3). One significant anomaly in the data, the drop in pH change from ~3.0 to ~1.2 at 6 days (for reactor systems with glucose and no graphite) might be due to the microbes feeding on each other after the glucose was depleted in the media.

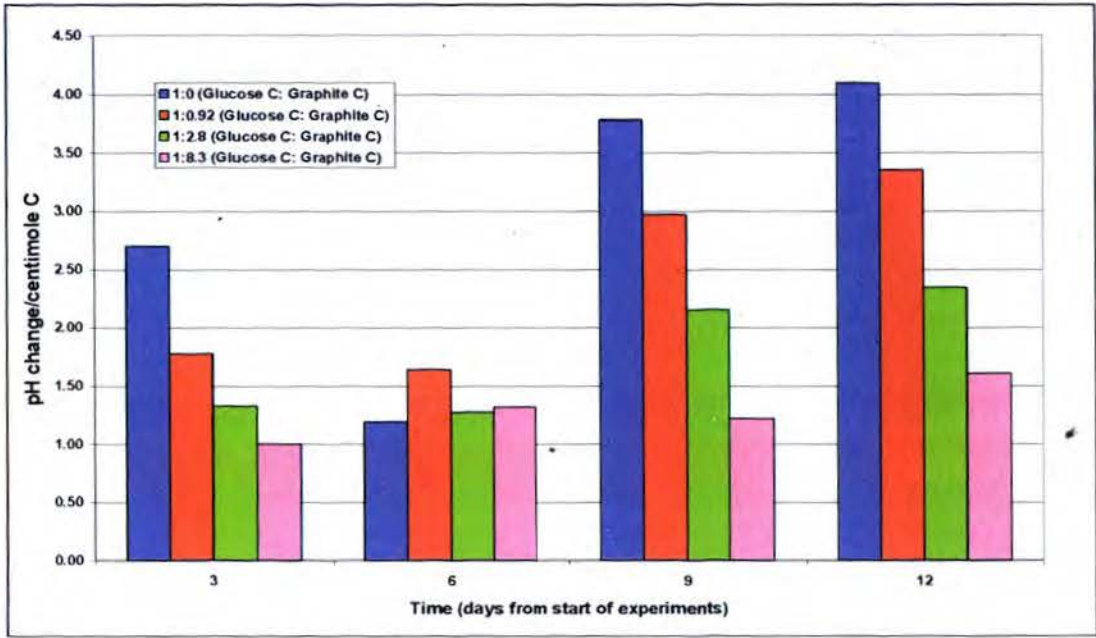


Figure 4.5: Cumulative pH change per centimole carbon as a function of time in the *Pseudomonas fluorescens* reactors

The growth pattern for the *Enterococcus* species (Figure 4.6) is different than other species, because it is gram-negative, which makes it unable to survive under harsh conditions. There is a larger pH change (above ~3.0) for reactor systems with no graphite and this reactor, along with the two reactors with intermediate graphite quantities, had fluctuating pH changes. The cumulative changes increased from day 3 to day 6, decreased between days 6 and 9, and increased to a cumulative high change value by day 12. In contrast, the reactor with least glucose (1:8.3 glucose C to graphite C) had a steadily increasing pH change, though the total change was smaller. In general, most pH change occurred by day 3 of reactor operation, with only incremental changes noted after day 3.

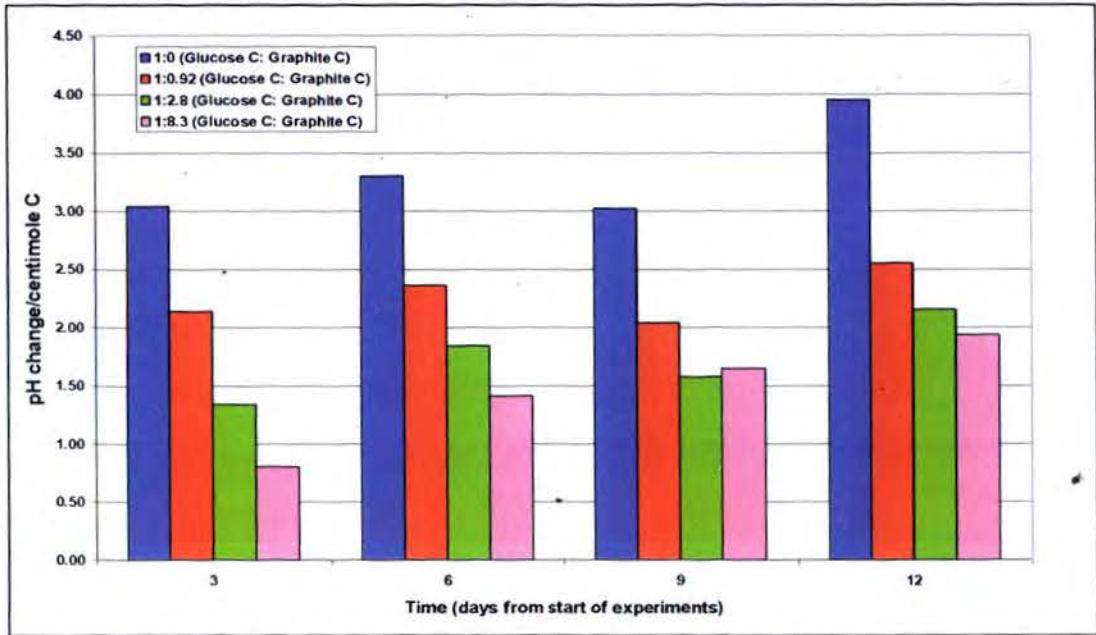


Figure 4.6: Cumulative pH change per centimole carbon as a function of time in the *Enterococcus* reactors

The mixed culture species has a different growth pattern as compared to the other species. The trend in ((pH change (Figure 4.7)) for reactor systems with glucose and no graphite starts off at ~3.7 (at 3 days), and gradually decreases until it stabilizes around 2.4 from 9 to 12 days. The stabilization might be because of the depletion of glucose in the reactor. pH changes in the graphite reactors also were most significant at day 3 and fluctuated incrementally to a smaller net change by day 12.

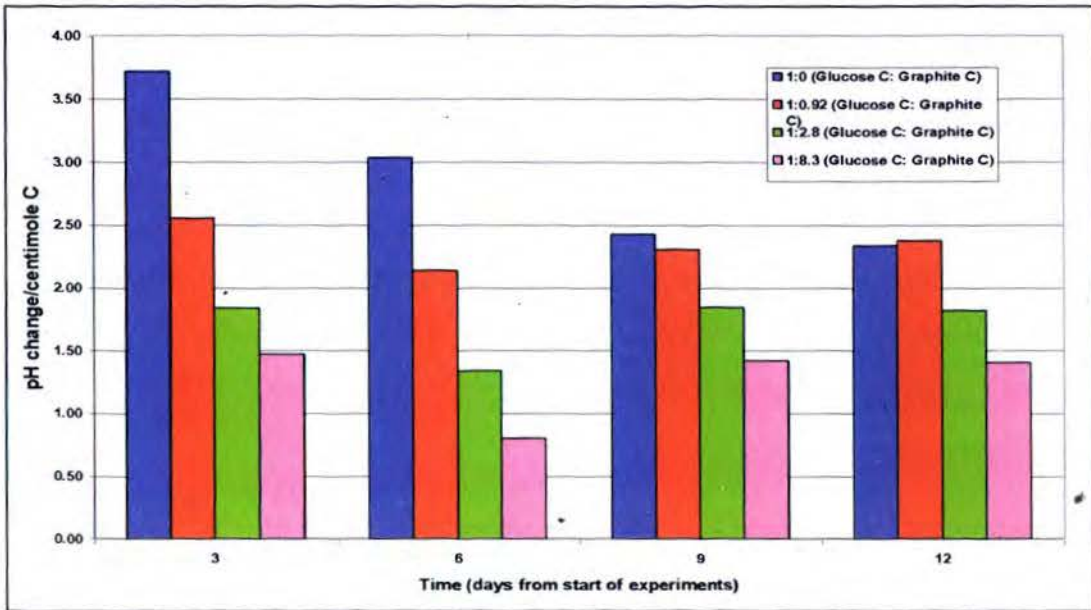


Figure 4.7: Cumulative pH change per centimole carbon as a function of time in the mixed culture species reactors

Each bacterial species grew differently with the different amounts of graphite in the systems; however, a consistent growth pattern was observed. Each bioreactor began operation at a near neutral pH ( $\sim 6.3$ ), which gradually decreased to an intermediate plateau (pH  $\sim 4.3$ ), and then further decreased through the 12-day experiment cycle time. The overall lowest bacterial growth was recorded in bioreactors containing a glucose carbon to graphite carbon ratio of 1:8.3. This relative lack of growth indicated that the quantity of glucose used in these systems was insufficient to obtain adequate growth in a graphite system.

#### 4.1.2 Flask reactors

Spectrophotometric analysis of the liquid medium is a more accurate indication of bacterial growth than pH. Visible light sent through a turbid sample (bacterial culture broth sample) is interpreted in the spectrophotometer as an absorbance value. The greater the bacterial cell numbers, the greater the absorbency values.

Use of small volume test tube reactors did not allow for spectrophotometric analysis. Therefore, a set of experiments were conducted in Erlenmeyer flasks instead of test tubes as bioreactors, where *Bacillus subtilis*, *Pseudomonas putida* and the mixed culture were used as inoculates in systems containing chunks ( $\sim 1 \text{ cm}^3$ ) of graphite rather than powder. These species were chosen because they exhibited better growth in the test-tube reactors in comparison to *Bacillus megaterium*, *Pseudomonas fluorescence* and *Enterococcus* species. A switch from graphite powder to monolith ( $\sim 1 \text{ cm}^3$  chunks) created a surface more amenable to post-reactor analysis. Illustrated in Figure 4.8 and Figure 4.9 are the bioreactors used during these experiments.



Figure 4.8: Bioreactors containing a graphite chunk and liquid medium without glucose. From Left 1) graphite only; 2) *Bacillus subtilis*; 3) *Pseudomonas putida*; 4) mixed culture.



Figure 4.9: Bioreactors containing a graphite chunk and liquid medium with glucose. From Left 1) Graphite only; 2) *Bacillus subtilis*; 3) *Pseudomonas putida*; 4) Mixed culture.

In Figure 4.8, the clarity of the solution in the *Bacillus subtilis* and *Pseudomonas putida* bioreactors indicates there was little or no growth. In contrast, the turbidity of the mixed culture bioreactor (0.707 absorbency (see Table 4.1)) indicates bacterial growth. Lack of growth in the single species systems may be explained by individual species being unable to reproduce in the absence of glucose. Growth in the mixed culture bioreactor may be due to a synergistic relationship among the different species of the bacterial consortia, whereby microbes were feeding off each other's metabolites. A collection of species may be able to consume the graphite carbon source and/or the graphite impurities using metabolite by-products from unrelated reactions.

Table 4.1: Measured absorbance values for flask reactors

Bacterial species	absorbance values	
	Flask with graphite and glucose	Flask with graphite and NO glucose
Mixed culture	2.5	0.707
<i>Pseudomonas putida</i>	1.559	0
<i>Bacillus subtilis</i>	0.767	0

The turbidity of the bioreactor solutions containing glucose (Figure 4.9) illustrates that there is growth in the *Bacillus subtilis* and *Pseudomonas putida* reactors. This growth is also indicated by absorbency values (Table 4.1) of 0.767 and 1.559, respectively. The mixed culture grew better than the individual species in experiments with glucose (2.5 absorbency) and without glucose (0.707 absorbency) in the media. The latter result reaffirmed the need for glucose to promote optimal growth in the bioreactor system, thereby addressing experimental objective (2) to identify the bacterial growth medium optimum for the system.

Based on these experiments, the mixed culture was chosen over the individual species to be used in subsequent experiments. This choice contributed to achievement of the first objective of these experiments, namely to test the compatibility of candidate bacteria with a graphite system.

## 4.2 Effects of sodium salts on bacterial growth

The third experimental objective was to create a bioreactor environment in which bacteria consume a source of surrogate  $^{14}\text{C}$  in graphite. Results of experiments conducted to address this objective are presented and discussed in sections 4.2 and 4.3.

### **4.2.1 Bacterial growth with NaCl replaced by the carbon-containing sodium salts**

Spectrophotometry was used to gauge bacterial growth in these experiments. Visible light (600 nm) absorbency measurements for bioreactor solutions were performed daily for 2 hours at 30-minute intervals. The sampling times were chosen according to the typical growth period of the bacteria. The objective was to carefully monitor the bacterial growth to determine how a change in the source of Na might affect the microbes.

Figure 4.10 presents absorbance data for the sodium chloride reactors on the basis of moles of sodium. In this form, the data can be meaningfully compared with results from the sodium acetate and sodium bicarbonate experiments, which contained different molar quantities of sodium.

In the NaCl system (Figure 4.10) absorbance per mole Na decreased with the increase of NaCl concentration, with the best bacterial growth (1.4 absorbency per mole sodium) recorded for the reactor containing 0.203 M NaCl medium. In the system with the lowest salt concentration (0.136 M), growth increased after the first day and stabilized after day 2. For the higher salt concentration systems, the bacterial population is stable until after day 2, when significant growth occurs. The delayed growth in these bioreactors is likely due to initial acclimatization of the bacteria to excess NaCl.

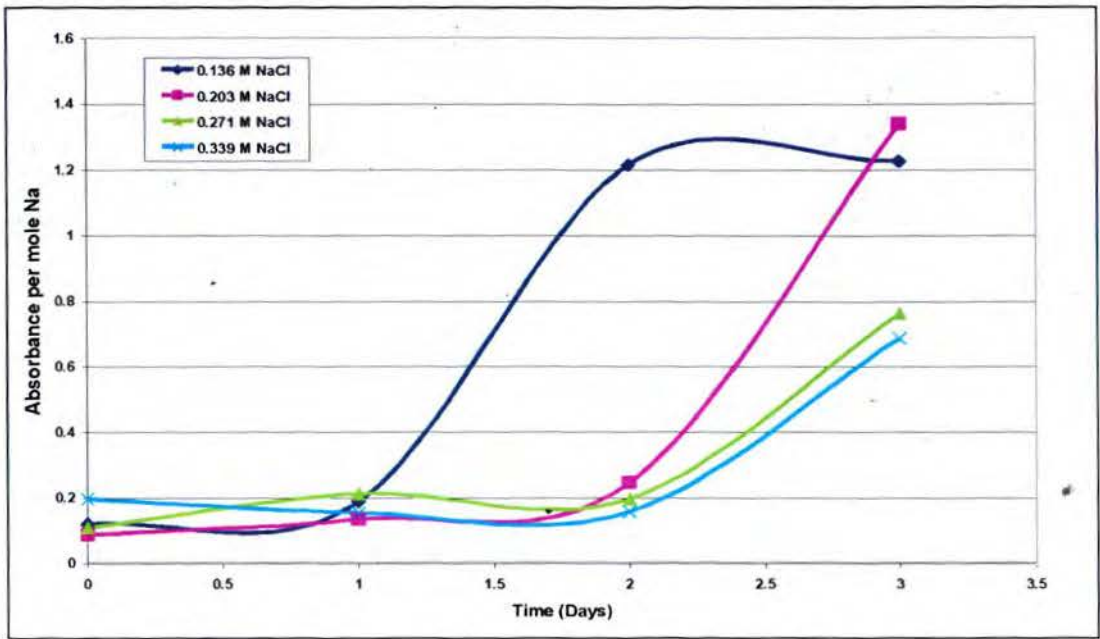


Figure 4.10: Absorbance per mole sodium for liquid media from bioreactors containing different concentrations of NaCl

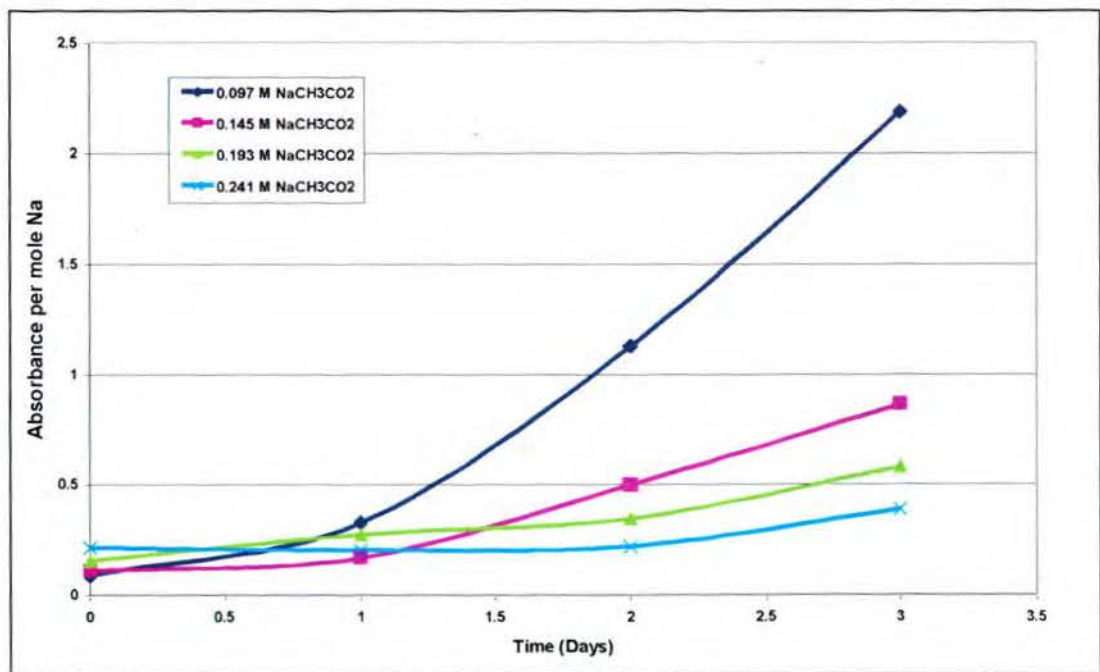


Figure 4.11: Absorbance per mole sodium for liquid media from bioreactors containing different concentrations of NaCH<sub>3</sub>CO<sub>2</sub>

In the bioreactors containing NaCl replacements, the most significant bacterial growth (Figure 4.11 and Figure 4.12) was observed when using 0.097 M  $\text{NaCH}_3\text{CO}_2$  and 0.094 M  $\text{NaHCO}_3$  in the medium. The higher concentrations of  $\text{NaCH}_3\text{CO}_2$  apparently had a negative effect on bacterial growth as indicated by the inverse relationship between absorbency and concentration in Figure 4.11. A similar correlation between growth and salt concentration is not indicated by absorbance data from the  $\text{NaHCO}_3$  or NaCl bioreactors; however, the higher salt concentrations generally appear to have a negative effect on bacterial growth as indicated by the inverse relationship between absorbency and concentration in the graphs.

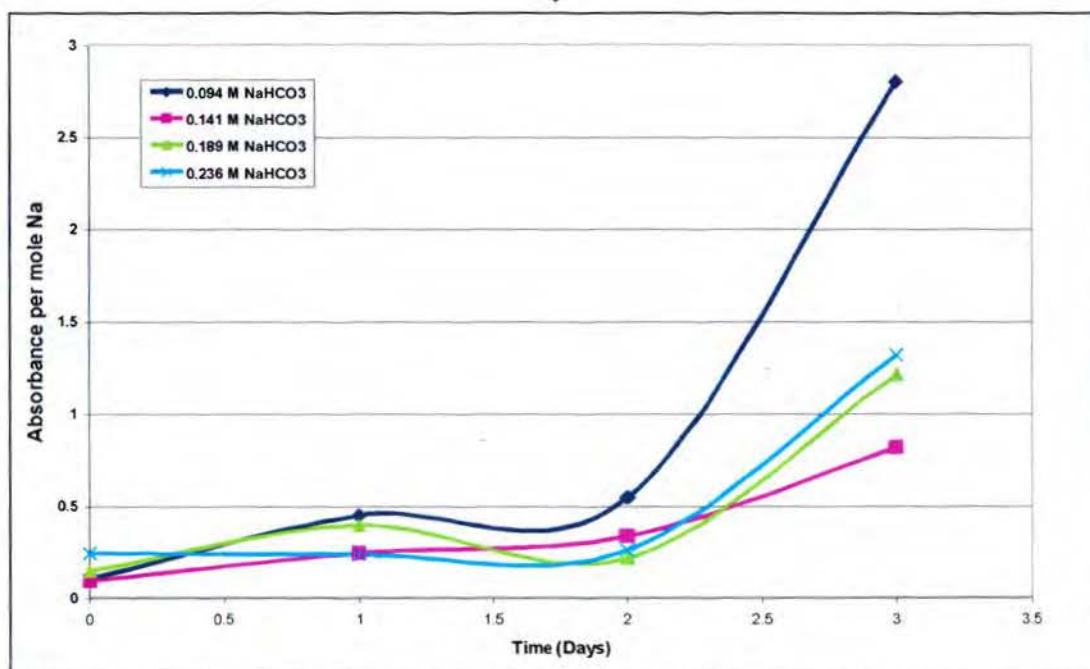


Figure 4.12: Absorbance per mole sodium for liquid media from bioreactors containing different concentrations of  $\text{NaHCO}_3$

## 4.2.2 Bacterial growth with glucose carbon replaced by the sodium salts carbon.

In the experiments discussed here, glucose carbon was replaced by carbon in  $\text{NaCH}_3\text{CO}_2$ ,  $\text{NaHCO}_3$ , or a mixture of the two salts. Reactors with 0% glucose and with 100% glucose (and no salt replacement) were operated to provide a basis for comparing results with those from the salt-replaced systems. The most significant growth, as expected, occurs in the system with 100% (0.6 M) glucose and the least growth with no glucose (See Figure 4.13). It is important to note, however, that although growth was less in the systems with salt and without glucose, there was growth.\* This fact seems to indicate metabolizing of the alternate C-source by bacteria.

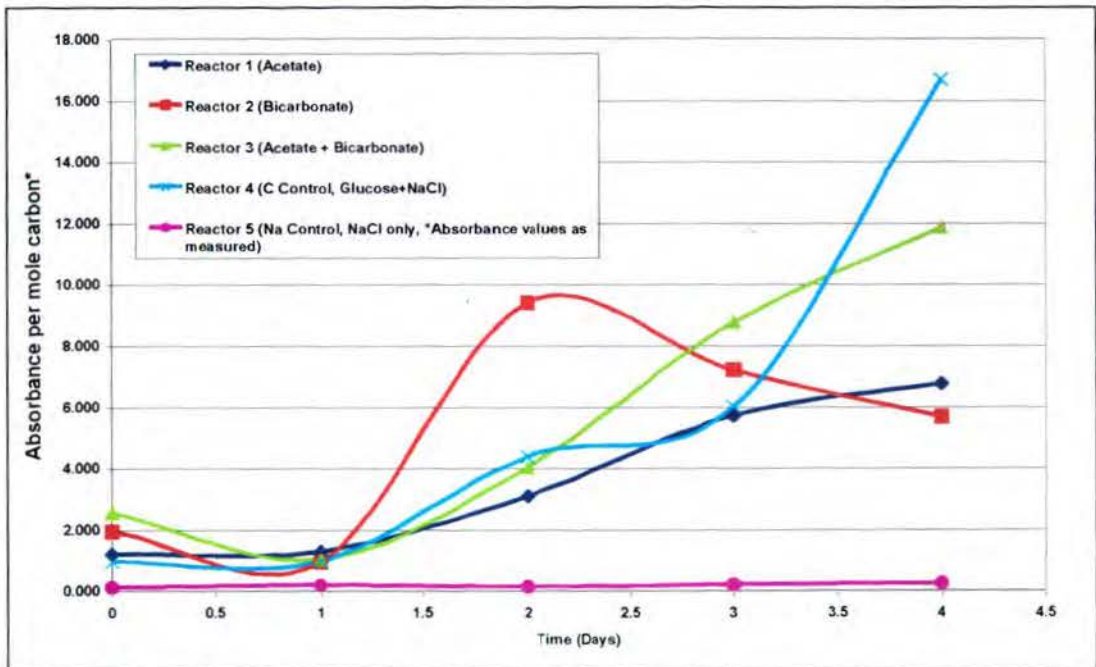


Figure 4.13: Absorbance curves for liquid media from bioreactors containing various  $\text{NaCH}_3\text{CO}_2$  to  $\text{NaHCO}_3$  to (0% or 100%) glucose molar ratios

For the salt-replaced systems, the most significant bacterial growth occurred in the reactor 3 system containing both acetate and bicarbonate salts at a molar ratio of approximately 2:1. At day 4 in this system, the absorbance per mole carbon was nearly 12. In the bioreactors containing only one salt, the day 4 values for absorbance per mole carbon were 6.8 and 5.7 for acetate and bicarbonate salts, respectively.

### 4.2.3 Bacterial growth with glucose and NaCl replacement by carbon-containing sodium salts

Illustrated in Figure 4.14 to Figure 4.16 are the absorbency plots from experiments conducted to determine a narrower range of values for the relative quantities of glucose and carbon-containing salts. The objective was to find the smallest quantity of glucose that would promote bacterial growth and bacterial metabolizing of alternate carbon sources, acetate and bicarbonate sodium salts.

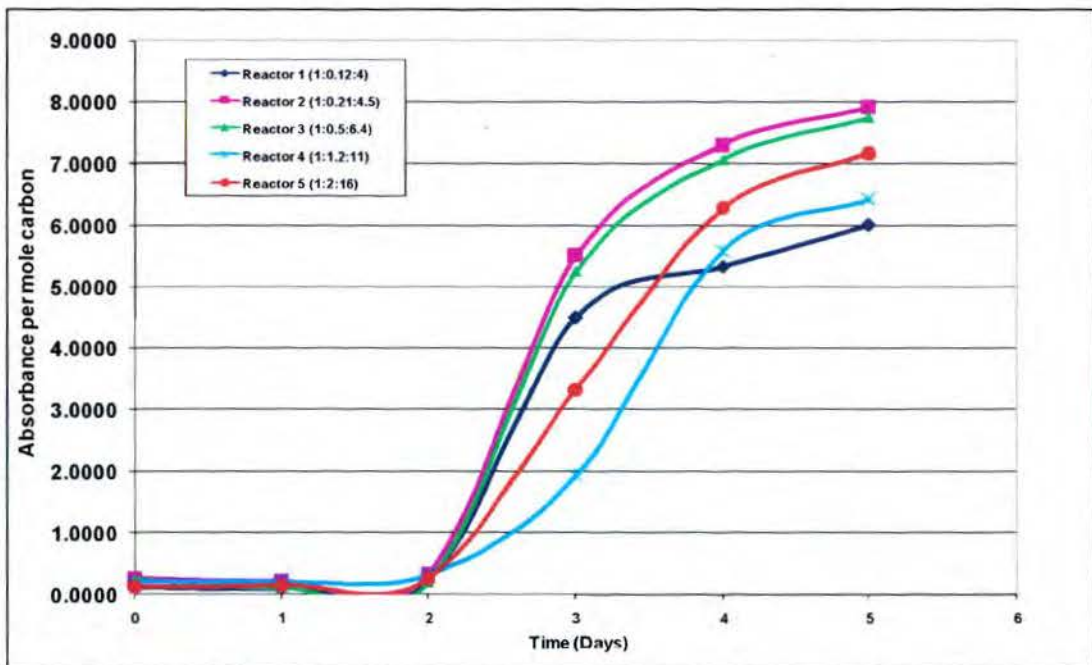


Figure 4.14: Absorbance per mole carbon in the system for liquid media from bioreactors containing various  $\text{NaCH}_3\text{CO}_2$  to  $\text{NaHCO}_3$  to (100%) glucose molar ratios

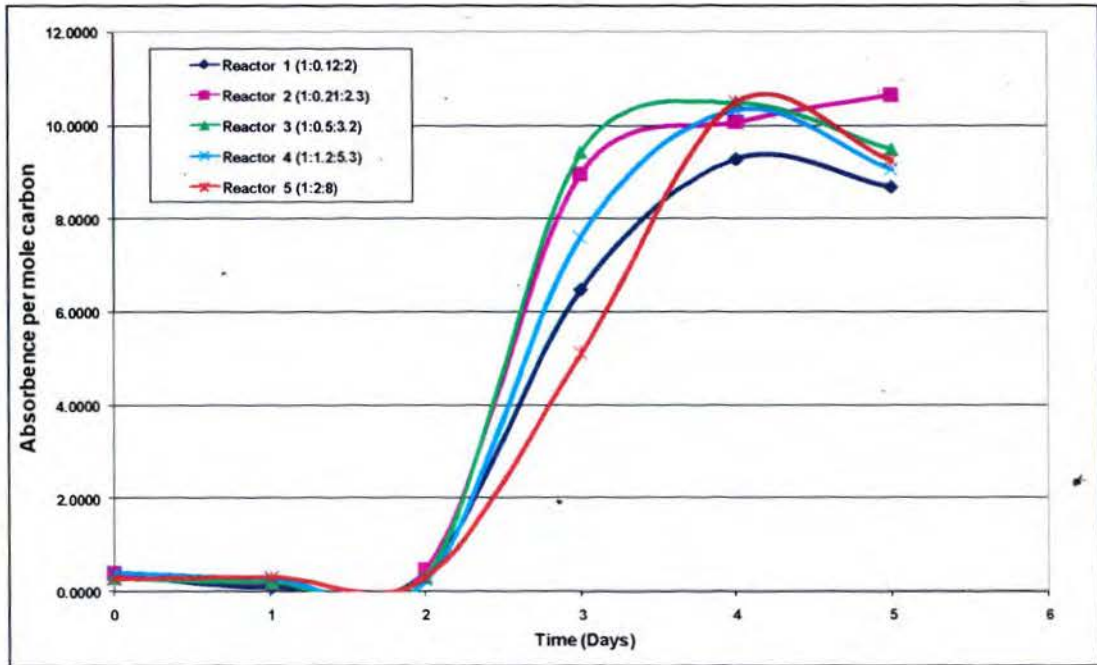


Figure 4.15: Absorbance per mole carbon in the system for liquid media from bioreactors containing various  $\text{NaCH}_3\text{CO}_2$  to  $\text{NaHCO}_3$  to (50%) glucose molar ratios

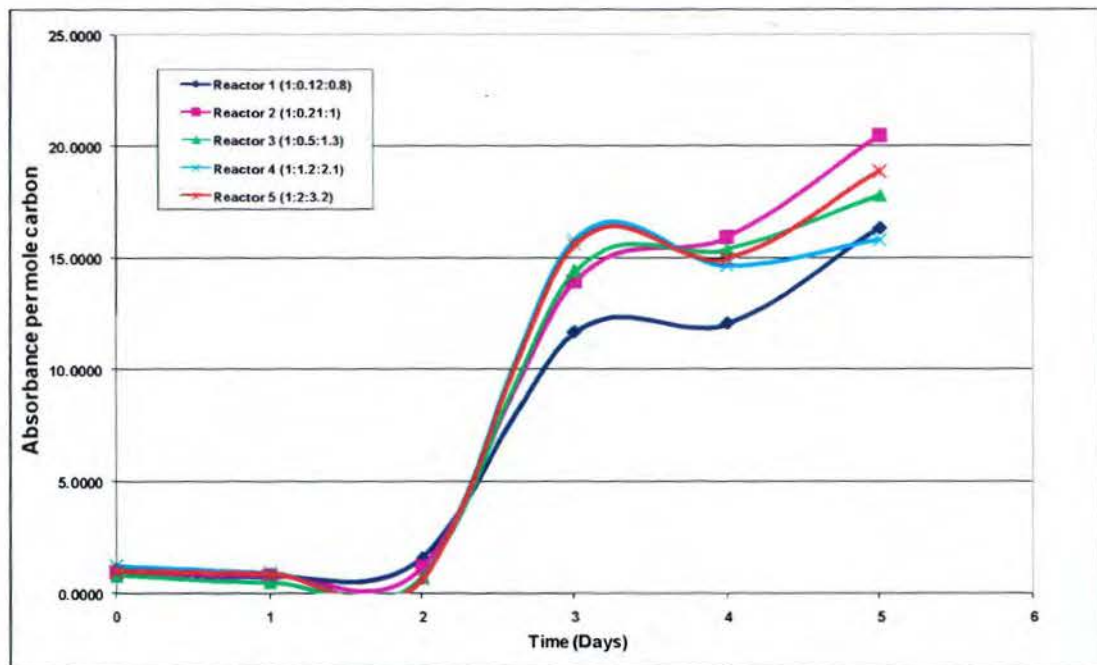


Figure 4.16: Absorbance per mole carbon in the system for liquid media from bioreactors containing various  $\text{NaCH}_3\text{CO}_2$  to  $\text{NaHCO}_3$  to (20%) glucose molar ratios

As previously noted, bacterial growth (increased absorbance) in systems without glucose indicate that bacteria may metabolize the sodium salts. General trends in Figure 4.14 to 4.16 indicate less growth (lower absorbency per mole carbon) in experiments conducted with little or no glucose in the system, which confirms that bacteria prefer glucose as (a carbon source) instead of the sodium salts. The most significant growth occurred in systems with more acetate carbon than bicarbonate carbon. This result is consistent with the previous experiments. As expected, bacterial growth occurred in a system with (100%) 0.6 M glucose. However, the system with (20%) 0.12 M glucose, had the most efficient bacterial growth (~20.5 absorbency per mole carbon), and was chosen for further experiments performed to gather evidence to reach the third objective.

### 4.3 Experiments with $^{14}\text{C}$ -labelled sodium salts

The presence of growth in experiments performed with the non-active carbon-containing salts was not direct evidence that bacteria processed these alternative C-sources. It is possible, for example, that bacteria grew using some unknown impurity in the bioreactor systems. As such, Objective 3 (to create a bioreactor environment in which bacteria consume a source of surrogate graphite  $^{14}\text{C}$ ) had yet to be achieved.

To gather the necessary evidence, experiments were performed with fractions of glucose replaced by radioactive  $^{14}\text{C}$ -labelled ( $\text{NaC}_2\text{H}_3\text{O}_2$  and  $\text{NaHCO}_3$ ) sodium salts. Figure 4.17 and Figure 4.18 illustrate the phase (gas, liquid, bacterial solid) location of  $^{14}\text{C}$  during bioreactor operation with  $^{14}\text{C}$ -labelled sodium acetate and sodium bicarbonate salts. As previously discussed, the bioreactor phases were analyzed via liquid scintillation counting.

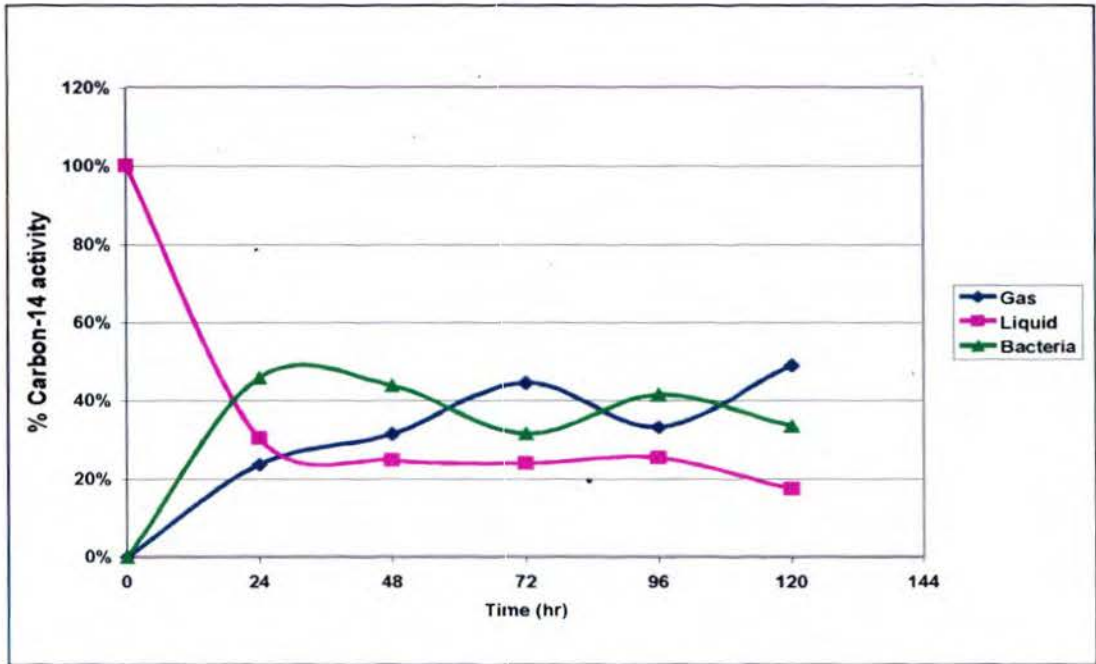


Figure 4.17: Activity distribution in bioreactors containing  $^{14}\text{C}$ -labeled  $\text{NaC}_2\text{H}_3\text{O}_2$  as a carbon source.

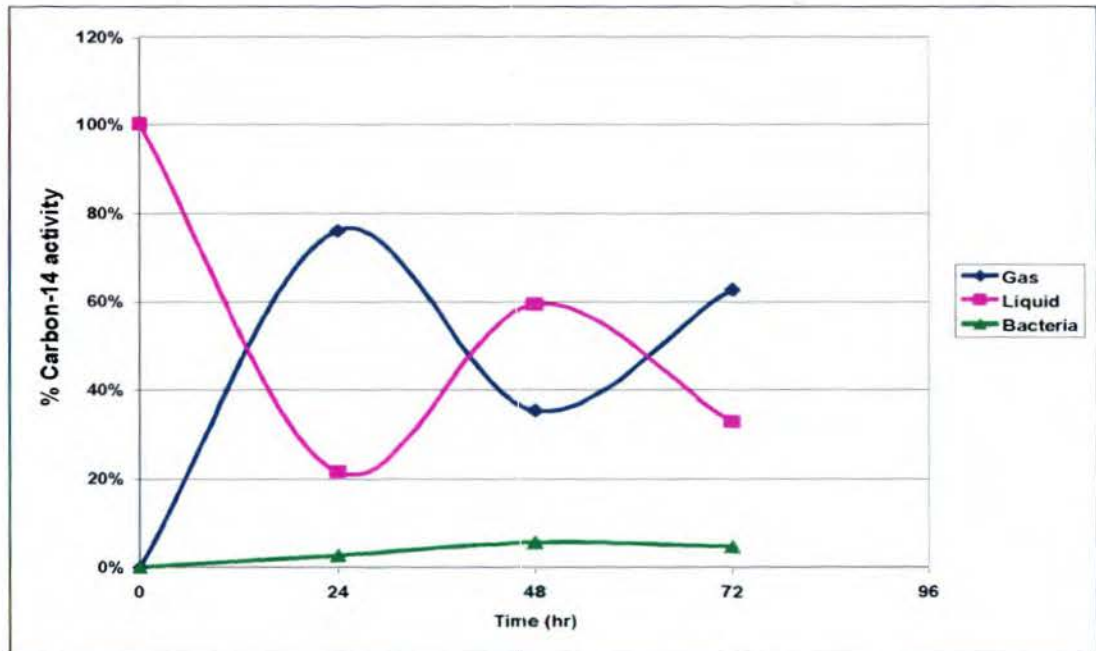
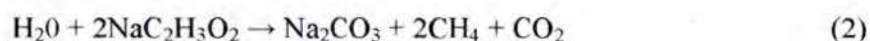


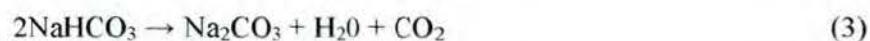
Figure 4.18: Activity distribution in bioreactors containing  $^{14}\text{C}$ -labeled  $\text{NaHCO}_3$  as a carbon source.

At time zero in the sodium acetate bioreactor system, (Figure 4.18) all the activity was contained in the liquid phase because the  $^{14}\text{C}$ -labelled  $\text{NaC}_2\text{H}_3\text{O}_2$  salt was dissolved in the growth medium. The bacteria consumed the  $\text{NaC}_2\text{H}_3\text{O}_2$  salt and proliferated as indicated by the increase in activity in the bacterial biomass (~46%) and the gas phase (~24%) during the first 24 hours. At the same time, activity decreased commensurately in the liquid phase to ~30%. The gradual decrease in activity between 24 and 72 hours in the bacterial biomass phase likely is due to depletion of sodium salt, which serves as a carbon source. Bacterial reproduction increases again as process metabolites become food sources. This phenomenon is marked by the increase in  $^{14}\text{C}$  activity in the bacterial biomass phase after 72 hours. The activity level in the liquid phase stays constant after the second day until the fourth.

The acetate ion in aqueous solution can chemically decompose to a carbonate ion, methane and carbon dioxide (equation 2) when heated. However, this reaction is not expected to occur under bioreactor system conditions. It is expected that sodium acetate is stable and remains in solution until affected by bacterial action.



Sodium bicarbonate does not have the same stability as sodium acetate in aqueous solutions. At the start of experiments with  $^{14}\text{C}$ -labelled  $\text{NaHCO}_3$ , all the activity was contained in the liquid phase as was the case for  $\text{NaC}_2\text{H}_3\text{O}_2$ . However, unlike the sodium acetate system, activity in the liquid phase decreased to ~21%, while only ~3% could be detected in the bacterial biomass phase. Activity in the gas phase increased to ~71% of the total system inventory. The high activity of the gas phase and low activity of the solid phase may be due to  $\text{NaHCO}_3$  chemical decay to  $\text{CO}_2$  (equation 3).



Results from experiments conducted with  $^{14}\text{C}$ -labelled sodium salts but no bacteria offer further evidence that bacteria consume the salts (Table 4.2). With no bacteria present, ~69% of the activity in the  $\text{NaC}_2\text{H}_3\text{O}_2$  experiments stayed in liquid phase at 24 hours, with the balance going to the gas phase. In contrast, the liquid phase of the  $\text{NaC}_2\text{H}_3\text{O}_2$  system charged

with bacteria contained ~30% of the  $^{14}\text{C}$  activity at 24 hours. In the  $^{14}\text{C}$ -labelled  $\text{NaHCO}_3$  system without bacteria, ~75% of the  $^{14}\text{C}$  activity was detected in the gas phase with the remainder in the liquid. This result further supports the theory of  $\text{NaHCO}_3$  decomposition due to chemical instability.

Table 4.2 provides information regarding  $^{14}\text{C}$  activity distribution between gas and liquid phases from control reactors operated without bacteria.

Table 4.2: Activity distribution (at 72 hours) in the  $^{14}\text{C}$ -labeled salt reactors with no bacteria

Acetate				
Phase	Counts per minute	Decays per minute	Total Activity (Bq)	% of Total Activity
Gas	3.0	4.0	35	31
Liquid	29.67	42.67	79	69
Bicarbonate				
Phase	Counts per minute	Decays per minute	Total Activity (Bq)	% of Total Activity
Gas	5.0	7.67	22	75
Liquid	3.0	4.0	7.4	25

## 5. CONCLUSION

The research project undertaken achieved its experimental objectives to: (1) test the compatibility of candidate bacteria with a graphite system, (2) identify the bacterial growth medium optimum for the system, and (3) create a bioreactor environment in which bacteria consume a source of surrogate  $^{14}\text{C}$  in graphite.

Based on the experiments performed with different bacterial species (objective (1)), the mixed culture bacterial consortium was chosen over the individual species to be used in subsequent experiments. This is because the mixed culture grew better than the individual species in experiments conducted with glucose (2.5 absorbency) and without glucose (0.707 absorbance) in the media. Experiments performed without glucose reaffirmed the need for glucose to promote optimal growth in the bioreactor system, which addressed experimental objective (2) to identify the bacterial growth medium optimum for the system.

Objective 2 was also addressed by experiments performed with the non-active carbon-containing salts to determine any significant effects of the salts on bacterial growth. The results confirmed that bacteria can grow in the presence of the salts, and that some glucose is needed to promote bacterial growth in a system. A system with 0.12 M glucose (20% of the typical quantity in a growth medium) was chosen for use in subsequent experiments. Bacterial growth in the presence of the salts was not a direct indication that the bacteria were able to process the salts as a food source alternative to glucose. As such, these experiments did not satisfy our third research objective.

To reach the third objective,  $^{14}\text{C}$ -labelled  $\text{NaC}_2\text{H}_3\text{O}_2$  and  $\text{NaHCO}_3$  were introduced to the bioreactor systems. The location of  $^{14}\text{C}$  was tracked before, during and after reactor operation to assess the processes that affected the salts. In all reactors containing  $^{14}\text{C}$  salts, 100% of the  $^{14}\text{C}$  was in the liquid phase at the start of reactor operation. The presence of activity in the bacterial biomass (~46%) and the gas phase (~24%) during the

first 24 hours of operation with  $\text{NaC}_2\text{H}_3\text{O}_2$  suggests that bacteria consumed the salt. Salt consumption was reaffirmed by the commensurate decrease (from 24 to 72 hours) of the total  $^{14}\text{C}$  inventory in the liquid medium (100% initially) to ~30%, where it was provided as an alternative (to glucose) food source for the microbes. In the bioreactor systems containing sodium bicarbonate, activity in the gas phase increased to ~71% of the total system inventory, while ~21% and ~3% of the  $^{14}\text{C}$  activity was detected in the liquid and the bacterial biomass phases, respectively.  $\text{NaHCO}_3$  seems to have little or no effect on bacterial growth, because unlike the acetate, bicarbonate is not stable in aqueous solution at reactor conditions.

Based on results of this research, it can be concluded that microbes can process (consume)  $\text{Na}^{14}\text{C}_2\text{H}_3\text{O}_2$  salt. This conclusion is supported by the shift of  $^{14}\text{C}$  content from liquid to solid phase (bacterial biomass) and/or gaseous phase (bioreactor off-gas). Implications of this conclusion may extend to application in bacterial remediation of irradiated graphite. Knowledge of the  $^{14}\text{C}$  chemical structure in irradiated graphite will facilitate the choice of an appropriate salt for experiments such as those performed in this project. The objective would be bacterial processing of a salt containing carbon with the same bond type as the bonding of  $^{14}\text{C}$  in irradiated graphite. Successful processing of such a salt would indicate that processing of the  $^{14}\text{C}$  counterpart in irradiated graphite is a possibility. The next research step would be experiments with neutron irradiated graphite.

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