EUROPEAN COMMISSION

Community Research

SEVENTH FRAMEWORK PROGRAMME

# CARBOWASTE

***Treatment and Disposal of Irradiated Graphite and Other Carbonaceous Waste***

**Grant Agreement Number: FP7-211333**

## Deliverable D-4.4.2

# Radiocarbon Reduction in graphite by Microbiological Treatment

Author(s):
Mary Lou Dunzik-Gougar, Peter J. Williams, Itumeleng I. Mafishane, and G. Ashy Pete
PBMR & University of Pretoria, RSA

Document Number: CARBOWASTE-D-1203-4.4.2
Date of issue of this report: 23/03/2012

Start date of project: **01/04/2008** Duration: **60 Months**

| **Project co-funded by the European Commission under the Seventh Framework Programme (2007 to 2011) of the European Atomic Energy Community (EURATOM) for nuclear research and training activities** | | |
|---|---|---|
| **Dissemination Level** | | |
| **PU** | Public | |
| **RE** | Restricted to the partners of the CARBOWASTE project | |
| **CO** | Confidential, only for specific distribution list defined on this document | **X** |

# Distribution list

| Person and organisation name and/or group | Comments |
|---|---|
| WP4 members | |
| Executive Board | |
| Co-ordinator | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |

| CARBOWASTE | | |
|---|---|---|
| **Work package:** 4<br>**Task: :** 4.2 | **CARBOWASTE document no:**<br>CARBOWASTE-1203-D-4.4.2.<br>(e.g. May 2008 as date of issue: 0805) | **Document type:**<br>D=Deliverable / M=Minutes /<br>P=Progress report /<br>T=Technical Report |
| **Issued by:** PBMR, RSA<br>**Internal no.:** CW1203-Deliverable-4-4-2-a | | **Document status:**<br>Final |

**Document title**

# Radiocarbon Reduction in graphite by Microbiological Treatment

**Executive summary**

Results of experiments described in this document indicate microbial remediation may be a suitable treatment option for irradiated graphite from nuclear reactors. The characterized microbe mixture was able to grow in the presence of, and on, inert graphite. Furthermore, the microbes processed $^{14}C$ target species present in a chemical form thought to be similar to that of $^{14}C$ compounds expected to form in irradiated graphite. Experiments with, and analysis of, irradiated graphite are necessary to determine the chemical form of $^{14}C$ and the viability of bacterial processing of that chemical in the radioactive environment. The actual chemical form(s) of $^{14}C$ in irradiated graphite may be a determining factor in the ultimate feasibility of such an application.

**Revisions**

| Rev. | Date | Short description | Author | Internal Review | Task Leader | WP Leader |
|---|---|---|---|---|---|---|
| 00 | dd/mm/yyyy | Issue | Name, Organisation | Name, Organisation *Signature* | Name, Organisation *Signature* | Name, Organisation *Signature* |
| 01 | 23/03/2012 | Final issue | M.L. Dunzik-Gougar PBMR | P.-J. Williams UoP | M.L. Dunzik-Gougar PBMR | von Lensa FZJ |
| | | | | | | |
| | | | | | | |
| | | | | | | |

# Table of Content

## Inhalt

# 1.0 Introduction

Graphite has been used as a moderator and reflector of neutrons in more than 100 nuclear reactors across Asia, Europe and North America. Due to their age and/or obsolescence, most of these reactors have been shutdown and many others are soon to follow. 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 $^{60}Co$, and the long-lived isotopes, principally $^{14}C$. 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 greater possibility of discharge into the biosphere (Mason and Bradbury, 1992).
Decommissioning of the reactors necessarily involves dismantling of irradiated graphite core components and management of this material as radioactive graphite waste. The worldwide inventory of irradiated graphite from these reactors is more than 230,000 metric tons (IAEA, 2006). Accordingly, proper management of this material is of interest on an international scale. One example of this growing interest is the CARBOWASTE research project of the European Commission's Framework 7, which has the sole aim to develop strategy and methods for irradiated graphite waste management for existing and future material.

Further, irradiated graphite management is of interest for reasons beyond the existing and decommissioned reactors (Generations I to III reactors). Indeed, deployment of Generation IV reactors will be greatly impacted by the existence of a technically and politically viable strategy for waste management. Implementation of the Very High Temperature Gas-cooled graphite core Reactor (VHTR), a Generation IV design, implies a need for an established method of irradiated graphite treatment for disposal and/or recycling.

Treatment for removal of radionuclides from irradiated graphite would allow disposal of the high volume, low activity material as lower level waste. Radionuclides collected from the treatment process could be concentrated in a suitable waste form for higher level, long-term disposal. Also, for serious consideration of recycling irradiated graphite components, the radioactivity of the material would need to be reduced considerably. This process would require removal of the majority of radioactive isotopes to reduce exposure to graphite manufacture workers during fabrication of new components from the recycled carbon/graphite. (EPRI, 2006)

Carbon-14 is a radionuclide of particular interest in the graphite as it is long-lived (5730-year half-life) and represents a significant percentage of the irradiated graphite activity after a short decay time (IAEA, 2006). As a low-energy β-emitter, $^{14}C$ 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.

Design of a $^{14}C$ removal method requires an understanding of the nature of $^{14}C$ formation in graphite, its distribution and its chemical form. $^{14}C$ is produced by three reactions in the reactor core:

$^{14}N + n \rightarrow {}^{14}C + p$ from trace nitrogen in the coolant gas and in graphite;
$^{17}O + n \rightarrow {}^{14}C + \alpha$ from trace oxygen in the coolant gas and in graphite;
$^{13}C + n \rightarrow {}^{14}C + \gamma$ from trace $^{13}C$ in graphite crystals and binder materials.

All available data from characterization of existing irradiated graphite indicate that the vast majority of $^{14}C$ is formed from the neutron activation of $^{14}N$ the graphite (Fachinger, 2006, IAEA, 2006, and Marsden et al, 2001). $^{14}C$ quantities produced from $^{17}O$ and $^{13}C$ reactions are negligible, because of the limited quantities of each isotope (0.04% $^{17}O$ and 1% $^{13}C$) and their relatively small thermal neutron absorption cross-section (0.24 barns for $^{17}O$ and 1.4 millibarns for $^{13}C$) (Khripunov et al., 2008). In contrast, 99.6% of nitrogen present in the graphite is $^{14}N$, which has a thermal neutron absorption cross section of approximately 2 barns.

Takahashi et al. (1992) suggest that a $^{14}C$ atom remains at the location of its formation, which is likely the location of the $^{14}N$ precursor diatomic molecule. Characterization of irradiated graphite from gas-cooled reactors has revealed that the highest concentration of $^{14}C$ is located in the outer surface layer of a component (Fachinger et al., 2007). Heat treatment (Fachinger, 2006 and von Lensa, 2008) and leaching (Takahashi et al., 2004) studies also have indicated high surface concentrations of $^{14}C$. These results are consistent with the surface adsorption of nitrogen after graphite billet manufacture.
It is suspected that the chemical form of $^{14}C$ in irradiated graphite is not crystalline graphite, but rather some more reactive form. The exact nature of $^{14}C$ chemical form(s) is unknown, but is being characterized by Dunzik-Gougar et al. (2008). Studies of irradiated graphite, as summarized by Marsden et al. (2002), suggest $^{14}C$ 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 $^{14}C$ in the graphite outer surface, where open pores may contain water and nitrogen impurities from air exposure.

The Electric Power Research Institute (2006) summarized options for treatment of irradiated graphite to remove radionuclides prior to disposal or recycle. The methods included chemical decontamination of the surface and thermal treatment with oxidizing gases. In either case, the objective would be removal of bulk $^{14}C$ activity from the outer surfaces of graphite components and from open porosity. Work presented in this paper explores a third possibility, namely biochemical treatment of irradiated graphite surfaces for removal of $^{14}C$.

Biochemical treatment is based on the principle of emulating naturally occurring processes to treat waste. Over billions of years, microorganisms have evolved mechanisms to survive in hostile environments and to adapt to changes in the environment. Microorganisms are also known to develop mechanisms to alter their environments to facilitate survival and reproduction. Microorganisms have been isolated that are capable of reducing the toxic forms of transuranic waste (TRU) to less mobile precipitable forms (Lloyd, 2003). Other cultures have demonstrated remarkable resistance to high radiation doses, although not doing much to treat the waste (Battista, 1997; White et al., 1999). More recently, cultures of melanising fungi from cold regions have been shown to utilise ionizing radiation for metabolism (Dadachova et al., 2007). These findings indicate the potential for application of biochemical treatment in conditions scientists once thought to be inhospitable to microorganisms.

## 2.0 Material and methods

A variety of bacteria were tested for compatibility with the experimental system, consisting of graphite and a growth medium solution. Five bacterial species (*Bacillus subtilis, Bacillus megaterium, Pseudomonas putida, Pseudomonas fluorescence* and *Enterococcus*) were chosen because they are environmental strains and have dormant stages, allowing them to 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 tested (to be discussed further later in this paper.)

The Štyriaková enrichment formula (Table 1) provided not only the medium for bacterial growth, but also the replacement (with graphite) of some portion of the standard carbon source, glucose..

Table 1. Composition of Štyriaková medium (Štyriaková, 2004)

| Component | Mass (g/L of distilled water) | Morality (mol/L) | Morality normalized with respect to carbon (in glucose) |
|---|---|---|---|
| $NaH_2PO_4$ | 0.42 | 0.0035 | 0.0058 |
| $(NH_4)_2SO_4$ | 0.80 | 0.0061 | 0.010 |
| NaCl | 0.19 | 0.0033 | 0.0054 |
| Glucose ($C_6H_{12}O_6 \cdot H_2O$) | 19.80 | 0.0999 | 0.17 |
| Carbon in glucose ($C_6H_{12}O_6$) | 7.2 | 0.60 | 1 |

The *Bacillus megaterium*, *Pseudomonas fluorescence* and *Enterococcus* species were quickly eliminated from consideration as their growth rates in a simple graphite-medium system was significantly lower than those of *Bacillus subtilis, Pseudomonas putida* and the mixed culture. For further elimination, the latter three options were used as inoculates in systems containing chunks (~1 $cm^3$) of graphite large enough to allow post-reactor surface analysis. Illustrated in Figures 1 and 2 are the bioreactors used during these experiments.

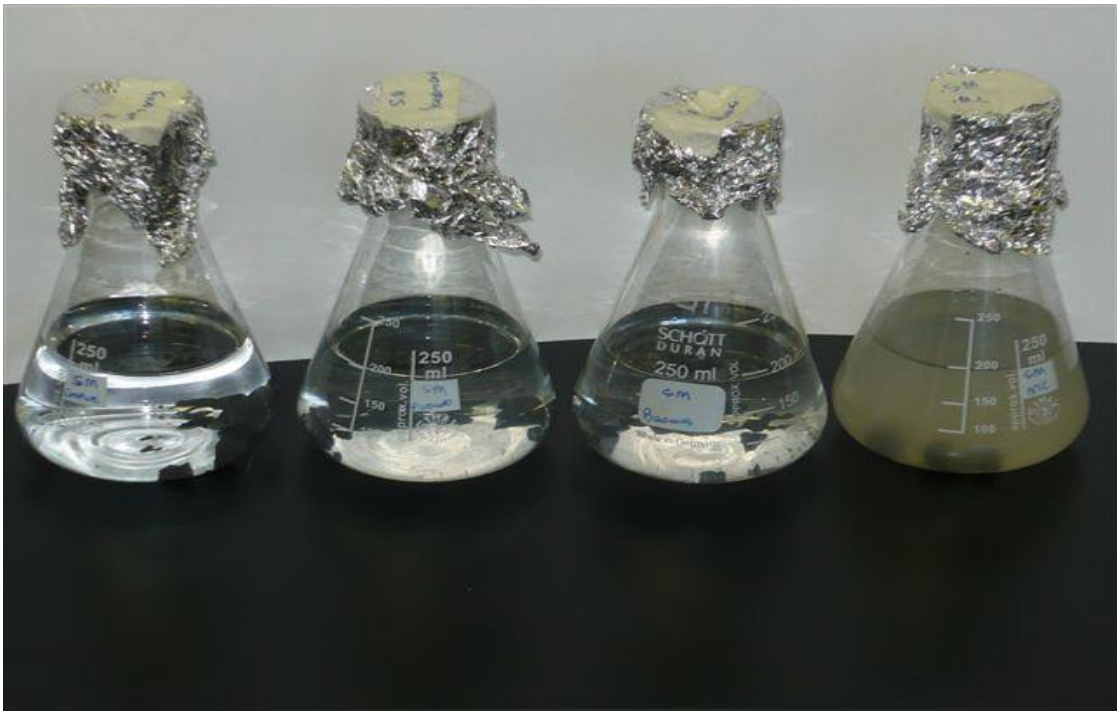



Figure 1: Bioreactors containing a graphite sample and liquid medium without glucose. From Left 1) graphite only; 2) *Bacillus subtilis*; 3) *Pseudomonas putida*; 4) mixed culture.


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

In Figure 1, the clarity of 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 2)) 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 2: 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 |
| *Pseudomonas putida* | 1.559 | 0 |
| *Bacillus subtilis* | 0.767 | 0 |

The turbidity of the bioreactor solutions containing glucose (Figure 2) illustrates that there is growth in the *Bacillus subtilis* and *Pseudomonas putida* reactors. This growth is also indicated by absorbency values (Table 2) 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.

Further, scanning electron microscopic (SEM) analyses of the graphite surfaces before and after operation of the mixed culture bioreactor indicate the presence of bio-film formation. Given the inert, and even inhospitable, nature of graphite, the creation of a bio-film on the material surface could not be assumed.

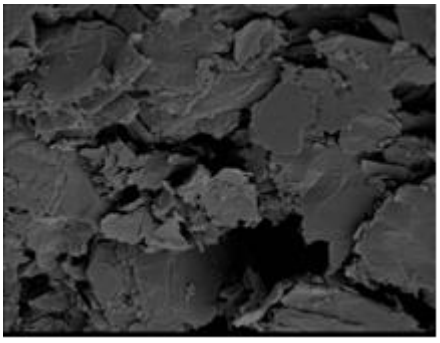
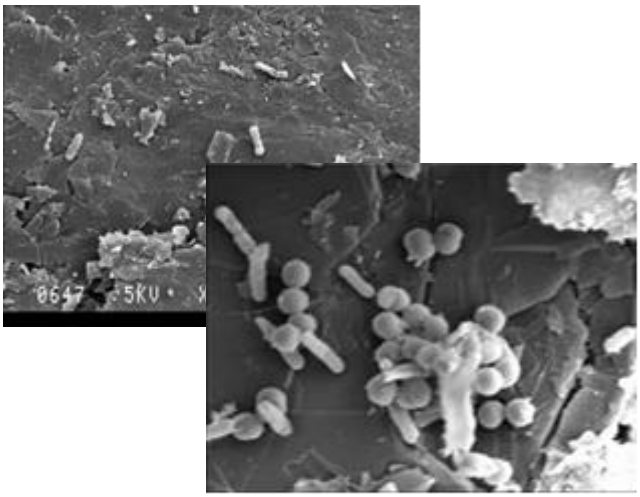


Figure 3: SEM image of graphite sample prior to bacteria exposure.



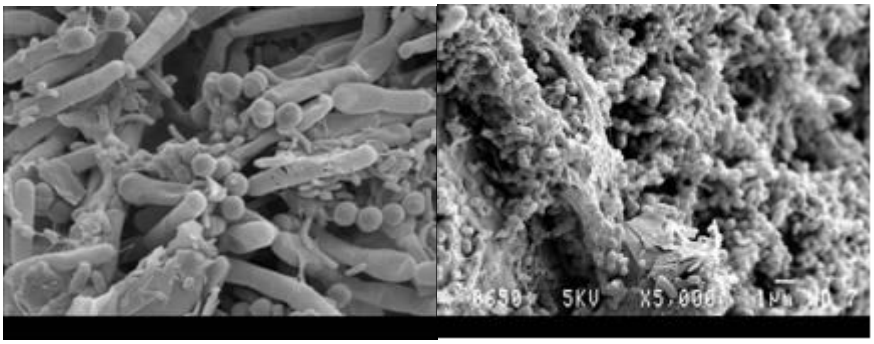


Figure 4: SEM images of post-bioreactor graphite surfaces with indications of significant biofilm presence (rod-like and spherical bodies).

The dominant species of the mixed culture were identified through the sequential processes of DNA extraction, Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) as detailed in a companion publication (Williams et al., 2010). Results of these analyses are summarized in Table 3.

Table 3: **Organisms identified from mixed cultures using denaturing gradient gel electrophoresis**

| **Organism identified** | **Isolated** | **Unique ability** |
|---|---|---|
| *Corynebacterium jeikeium* | *Corynebacterium* species have bee isolated from amniotic membranes and spent nuclear pools (Sing *et al.*, 2006) | Radiation resistant microorganisms. Able to oxidize C2-C4 alkanes (Aviam *et al.*, 2004). |
| *Corynebacterium auriscanis* | | |

| | | |
|---|---|---|
| *Clostridium acetobutylicum*<br>*Clostridium putrefaciens* | *Clostridium* species have been isolated from different environments such as: soil, decomposing biological material, lower gut of mammals and waste water (Suresh *et al.*, 2007) | *Clostridium* specie has been used in bioremediation approaches to treat nitroaromotic contaminants (Sembries and Crawford 1997). |
| *Bacillus subtilis*<br>*Bacillus pumilus* | *Bacillus* species have been isolated from a nuclear power plant (Chicote *et al.*, 2004). | n-alkane utilizing strain (Wentzel *et al.*, 2007) |
| *Pseudomonas putida* | *Pseudomonas* species have been isolated from nuclear power plant reactor contaminated with metals (Chicote *et al.*, 2004).<br>Gleeson *et al*. (2006) has isolated *Pseudomonas* on weathered K-feldspar. | Patent has been registered in which this organism is used to treat precious metals which contains refractory carbon (United States Patent 5244493, Brierley and Kulpa). |
| *Stenotrophomonas* | Constructed wetland (Nicomrata *et al.*, 2008), crude-oil samples (Yoshida *et al*., 2005), anaerobic reactor (Assih *et al*., 2002), and soil (Emerson and Moyer 1997) | Able to degrade high molecular weight polycyclic aromatic hydrocarbons (Boonchan *et al.*, 1998). Organism is able to degrade ethylene glycol, which is a toxic byproduct from polyester hydrolysis (Kim *et al*., 2001). |
| *Comamonas spp* | Species isolated from soil and a spent nuclear pool (Chicote *et al.*, 2004) | Possibly radiation resistant (Chicote *et al.*, 2004) |
| *Cupriavidus basilensis* | Isolated from environmens with high heavy metal contaminants (Nies 1999). | Able to degrade xenobiotics in the presentce of high heavy metal concentrations (Springael *et al*., 1993). |
| *Burkholderia spp* | *Burkholderia* species have been isolated from various environments: soil (LiPuma *et al*., 2002), crude-oil samples from Japanese oil stockpiles (Yoshida *et al*., 2005); human patients suffering from cystic fibrosis (LiPuma *et al*.,2002) and iron ore (Valverde *et al*., 2006) | Showed to be able to fractionate carbon and hydrogen isotopes |
| *Ralstonia pickettii* | Species of *Ralstonia* have been isolated from spent nuclear pools | |

## 2.1 Introduction of carbon-containing salts

The main objective of the experiments was to create a bioreactor environment in which bacteria consume a source of surrogate $^{14}C$ representing $^{14}C$ in irradiated graphite. The choice of a chemical surrogate for $^{14}C$ in graphite was difficult because little is known of its chemical bonding. As previously discussed, there is evidence suggesting $^{14}C$ 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 $^{14}C$ in the irradiated graphite outer surface, where open pores may contain water, oxygen and nitrogen from air exposure. Two salts with carbon-containing anions ($NaCH_3CO_2$ and $NaHCO_3$) were chosen to represent $^{14}C$ 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 $^{14}C$ on the graphite surface after irradiation. In the acetate and bicarbonate anions, 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.

## 2.2 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 (NaCl) with growth in medium containing alternate sources of sodium, the carbon-containing salts. Sodium is required for bacterial growth but too much can be harmful. During these experiments, Štyriaková medium without NaCl was prepared according to Table 1. 250 ml of the prepared medium was added to each 500 ml Erlenmeyer flask "reactor" along with one of three salts ($NaCH_3CO_2$, $NaHCO_3$ and NaCl). Four reactor sets correspond to 4 salt masses (Table 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 µl 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 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 | | | |
| $NaCH_3CO_2$ | 0.097 | 0.145 | 0.193 | 0.241 |
| $NaHCO_3$ | 0.094 | 0.141 | 0.189 | 0.236 |
| NaCl (Control) | 0.136 | 0.203 | 0.271 | 0.339 |

## 2.3 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 according to Table 5. Each bioreactor (500-ml Erlenmeyer flask with 250 ml of aqueous medium) was inoculated with 200 µl 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 5: Bioreactor carbon (and NaCl) content for glucose replacement experiments

| Bioreactor | Carbon concentration (moles/L medium) | | | Na concentration (moles/L medium) |
|---|---|---|---|---|
| | $NaCH_3CO_2$ | $NaHCO_3$ | 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 |

## 2.4 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 Tables 6 to 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 6: Glucose, $NaCH_3CO_2$ and $NaHCO_3$ concentrations in the 100% glucose bioreactor system for carbon and sodium replacement experiments

| Bioreactor (Acetate C : Bicarbonate C : Glucose C) | Concentrations | | | | | |
|---|---|---|---|---|---|---|
| | $NaCH_3CO_2$ | | $NaHCO_3$ | | 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 7: Glucose, $NaCH_3CO_2$ and $NaHCO_3$ concentrations in the 50% glucose bioreactor system carbon and sodium replacement experiments

| Bioreactor (Acetate C : Bicarbonate C : Glucose C) | Concentrations | | | | | |
|---|---|---|---|---|---|---|
| | $NaCH_3CO_2$ | | $NaHCO_3$ | | 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 8: Glucose, $NaCH_3CO_2$ and $NaHCO_3$ concentrations in the 20% glucose bioreactor system carbon and sodium replacement experiments

| Bioreactor (Acetate C : Bicarbonate C : Glucose C) | Concentrations | | | | | |
|---|---|---|---|---|---|---|
| | $NaCH_3CO_2$ | | $NaHCO_3$ | | 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 |

## 2.5 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 impeded growth; however, the presence of growth was not necessarily an indication that the salts facilitated 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 $^{14}C$ 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 $^{14}C$-labelled $NaC_2H_3O_2$ and $NaHCO_3$. Tracking the location of $^{14}C$ before, during and after reactor operation would provide information regarding the type of processes affected on the salts. The total $^{14}C$ 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 $^{14}C$ content from liquid to solid phase (bacterial biomass) and/or gaseous phase (bioreactor off-gas) may indicate microbial processing of the $^{14}C$ salt.

### 2.5.1 Experimental set-up

Figure 5 illustrates the configuration of the experimental apparatus. 500-ml Erlenmeyer flasks containing the growth medium, $^{14}C$-labelled salt and the microbial culture inoculum were used as bioreactors. Because it was crucial to account for all $^{14}C$ in the system, the apparatus was designed to oxidize the off-gas ($CH_4$, CO and $CO_2$) produced by bacteria during experiments to $CO_2$ (and $H_2O$). Regulated carbon-free-air carried the off-gas to a tube furnace, where it was oxidized, and the resulting $CO_2$ was trapped in a basic (1M NaOH) solution.

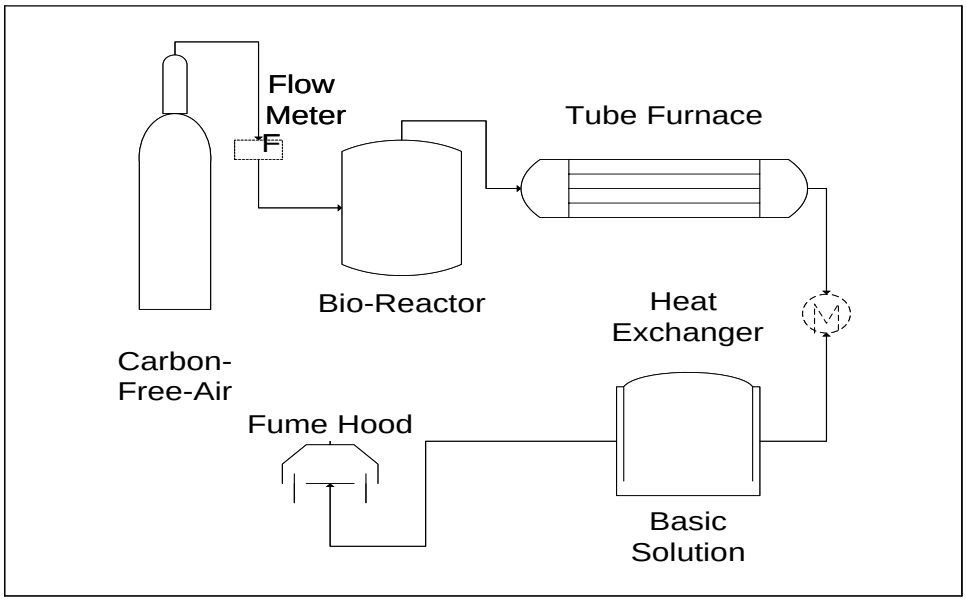



Figure 5: Schematic layout of the experimental set-up

### 2.5.2 Experiments with $^{14}$C-labelled sodium acetate

During these experiments 250 ml of Štyriaková growth medium was prepared as in Table 1 but without NaCl and with 20% of the usual glucose (0.12 M with respect to carbon). Na was provided (in the medium) via $^{14}$C-labelled sodium acetate. Also added were 1.7 ml of the $^{14}$C-labelled $NaCH_3CO_2$ (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) 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 9. The times were chosen with a range to reflect that used in experiments performed with non-active materials, for which reactors were operated from 24 to 120 hours.

Table 9: Bioreactor operation times

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

### 2.5.3 Experiments with 14C-labelled sodium bicarbonate

These experiments were conducted as described for $^{14}C$-labelled $NaCH_3CO_2$, with the exception that the salt used was $^{14}C$-labelled $NaHCO_3$, 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 $Na^{14}CH_3CO_2$. 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 $^{14}C$-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.

### 2.5.4 Liquid scintillation counting sample preparation

Samples from all three bioreactor phases (solid, liquid and gas) were analyzed for $^{14}C$ 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 25ºC 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).
$^{14}C$ content in the gas phase was determined via analysis of the NaOH solutions, which collected the $CO_2$ produced from reactor off-gas oxidation. Hionic-Fluor LSC cocktail, chosen for its compatibility with the basic solution, was mixed with the NaOH gas collection samples.

# 3.0 Results and Discussion

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

Spectrophotometry was used to gauge bacterial growth in reactors used for the salt replacement 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 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) 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.

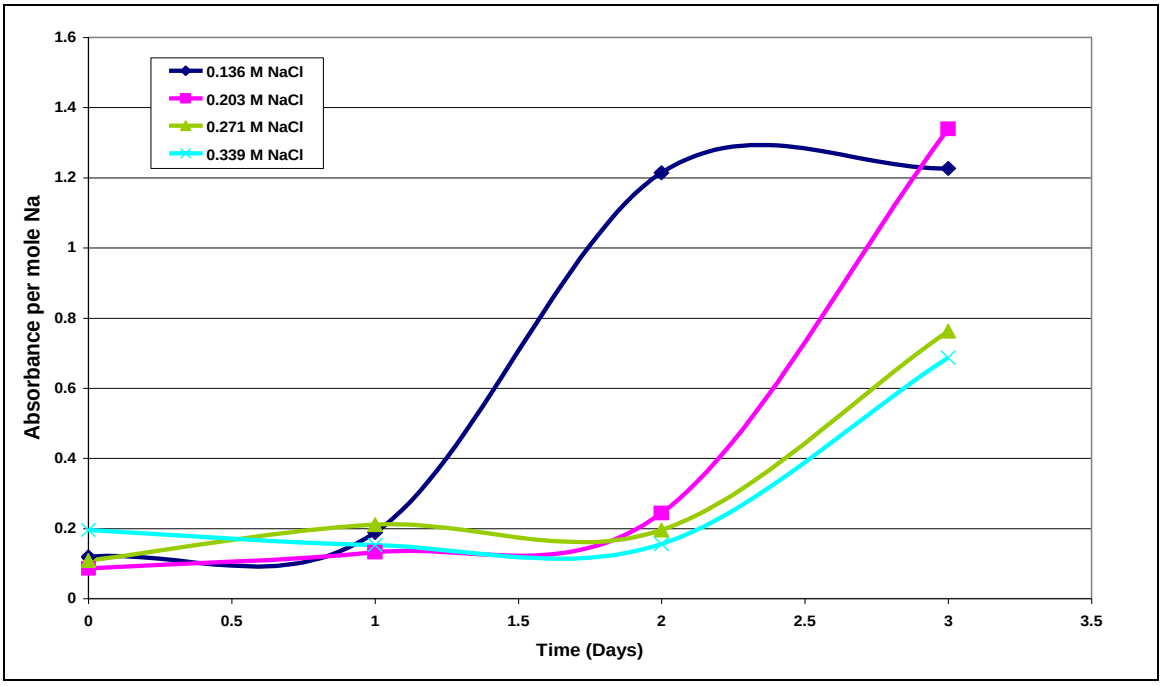



Figure 6. Absorbance per mole sodium for liquid media from bioreactors containing different concentrations of NaCl

In the bioreactors containing NaCl replacements, the most significant bacterial growth (Figures 7 and 8) was observed when using 0.097 M $NaCH_3CO_2$ and 0.094 M $NaHCO_3$ in the medium. The higher concentrations of $NaCH_3CO_2$ apparently had a negative effect on bacterial growth

as indicated by the inverse relationship between absorbency and concentration in Figure 5. A similar correlation between growth and salt concentration is not indicated by absorbance data from the $NaHCO_3$ 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.

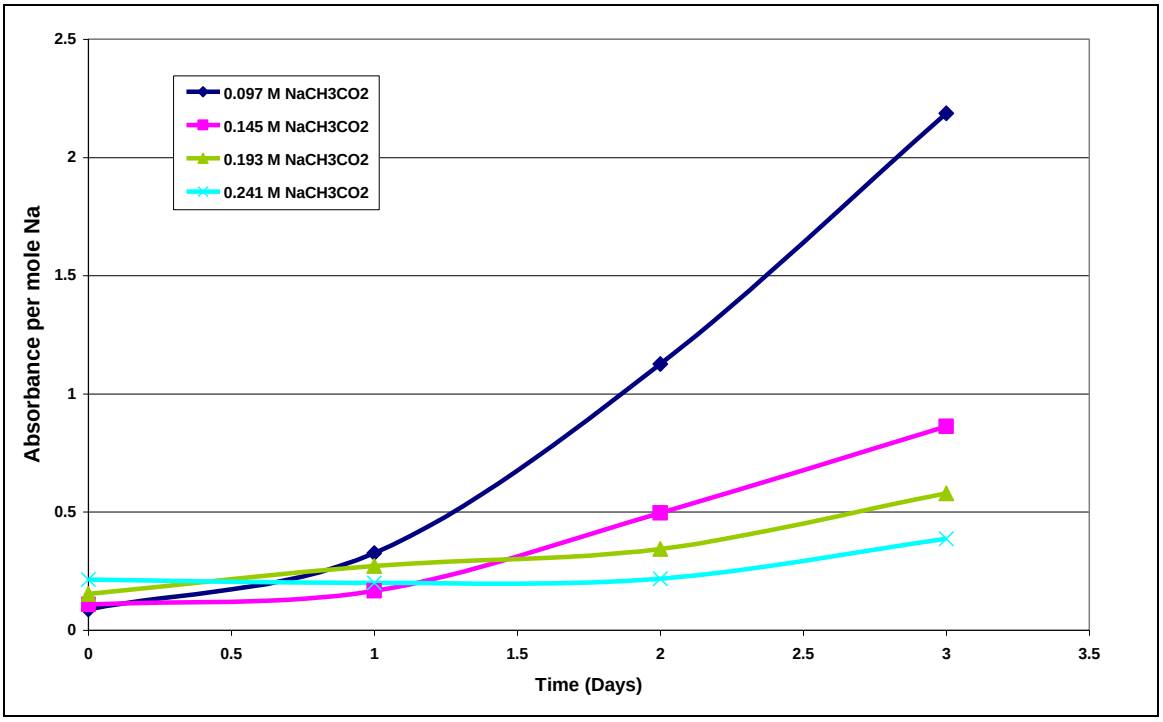



Figure 7. Absorbance per mole sodium for liquid media from bioreactors containing different concentrations of $NaCH_3CO_2$

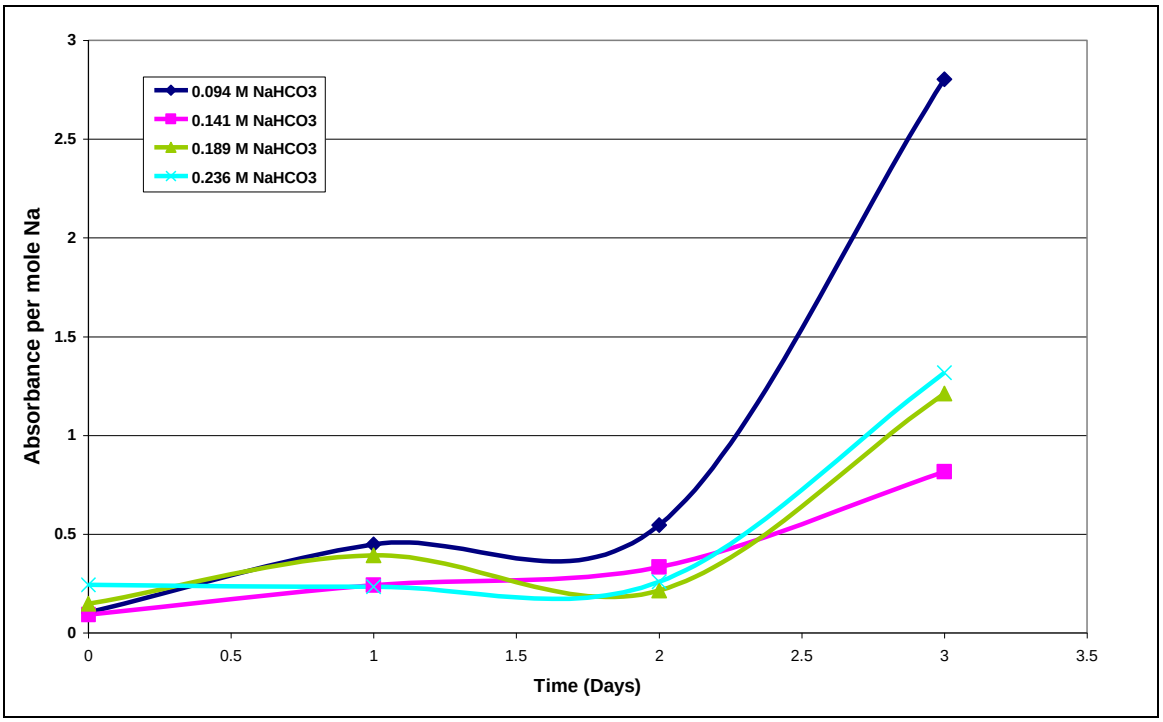



Figure 8. Absorbance per mole sodium for liquid media from bioreactors containing different concentrations of $NaHCO_3$

3.2 Bacterial growth with glucose carbon replaced by carbon-containing sodium salts

In the experiments discussed here, glucose carbon was replaced by carbon in $NaCH_3CO_2$, $NaHCO_3$, 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 9). It is important to note, however, that although growth was less in the systems with salt and without glucose, growth was still observed. This fact seems to indicate metabolizing of the alternate C-source by bacteria.

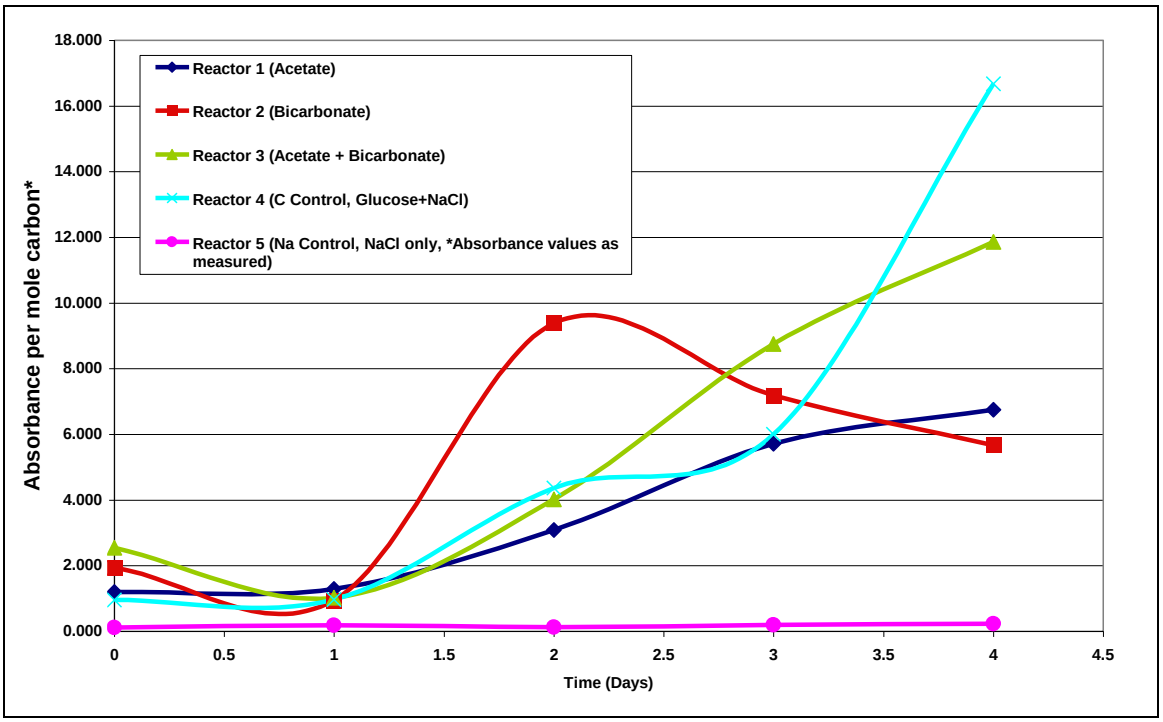



Figure 9: Absorbance curves for liquid media from bioreactors containing various $NaCH_3CO_2$ to $NaHCO_3$ to (0% or 100%) glucose molar ratios

For the carbon-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 contrast, 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.

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

Illustrated in Figures 8 to 10 are the absorbency plots from experiments conducted to determine a narrow, operational 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.

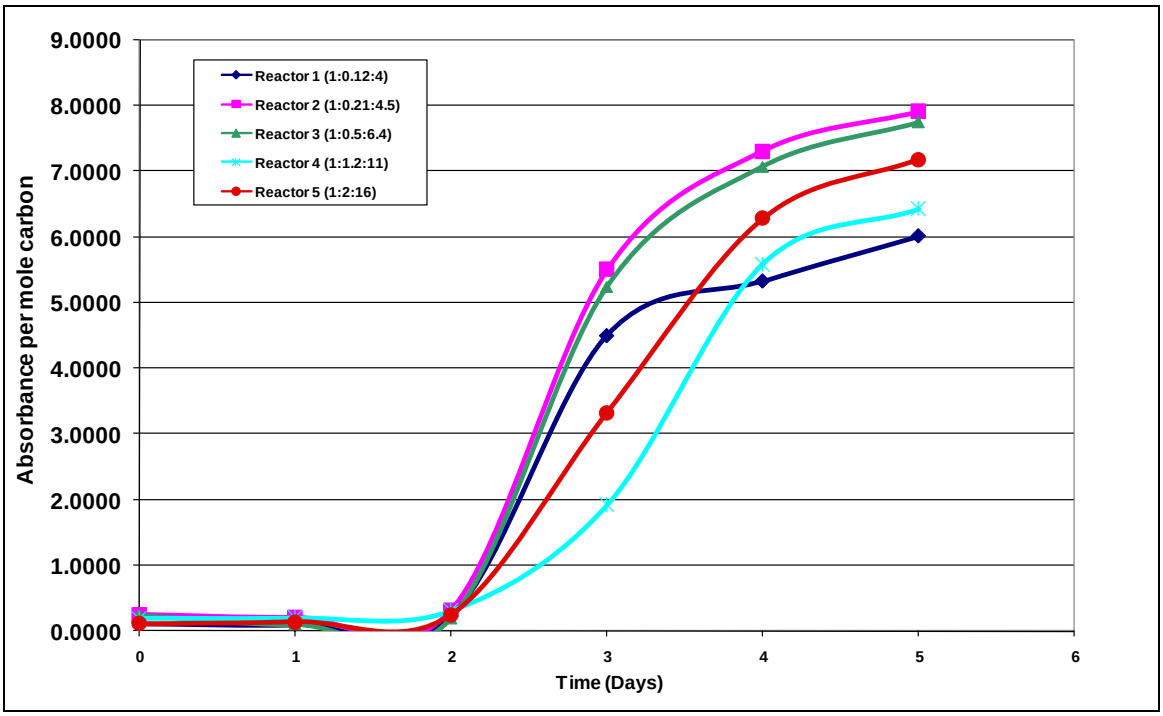



Figure 10: Absorbance per mole carbon in the system for liquid media from bioreactors containing various $NaCH_3CO_2$ to $NaHCO_3$ to (100%) glucose molar ratios

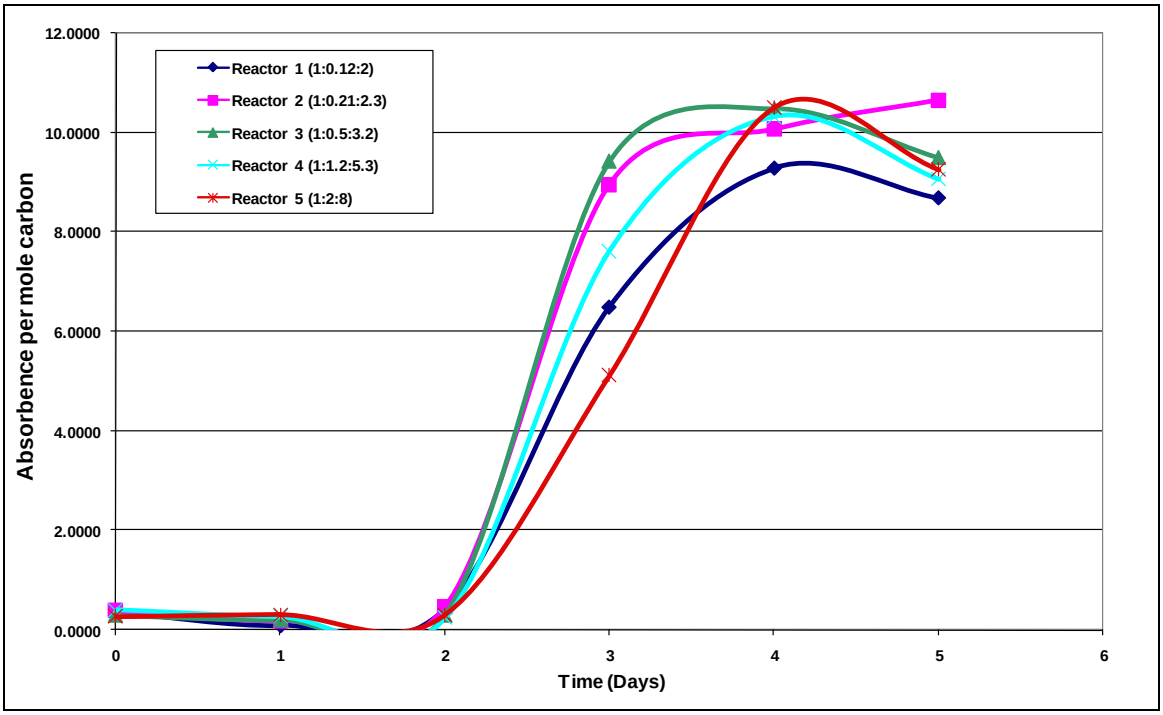



Figure 11: Absorbance per mole carbon in the system for liquid media from bioreactors containing various $NaCH_3CO_2$ to $NaHCO_3$ to (50%) glucose molar ratios

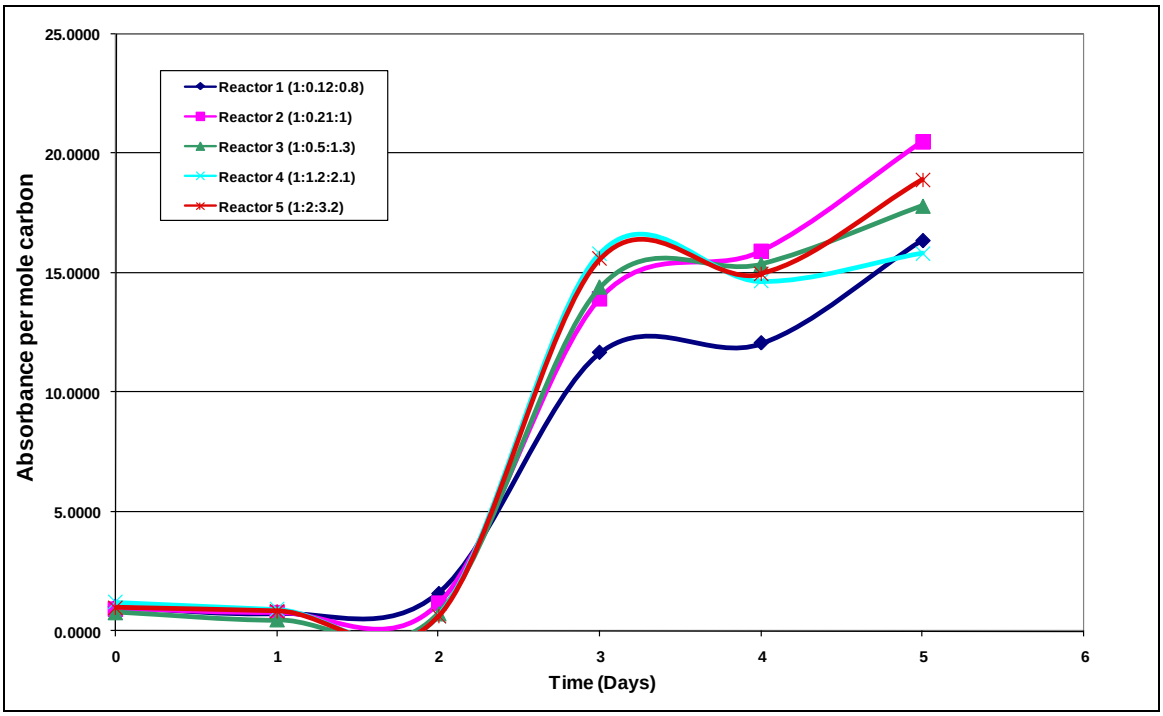



Figure 12 Absorbance per mole carbon in the system for liquid media from bioreactors containing various $NaCH_3CO_2$ to $NaHCO_3$ to (20%) glucose molar ratios

As previously noted, bacterial growth (increased absorbance) in systems without glucose indicate that bacteria may metabolize the sodium salts. General trends in Figures 9 to 12 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.

## 3.4 Experiments with 14C-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, the objective to create a bioreactor environment in which bacteria consume a source of surrogate graphite $^{14}C$ had yet to be achieved.
To gather the necessary evidence, experiments were performed with fractions of glucose replaced by radioactive $^{14}C$-labelled ($NaC_2H_3O_2$ and $NaHCO_3$) sodium salts. Figure 13 and Figure 14 illustrate the phase (gas, liquid, bacterial solid) location of $^{14}C$ during bioreactor operation with $^{14}C$-labelled sodium acetate and sodium bicarbonate salts. As previously discussed, the bioreactor phases were analyzed via liquid scintillation counting.

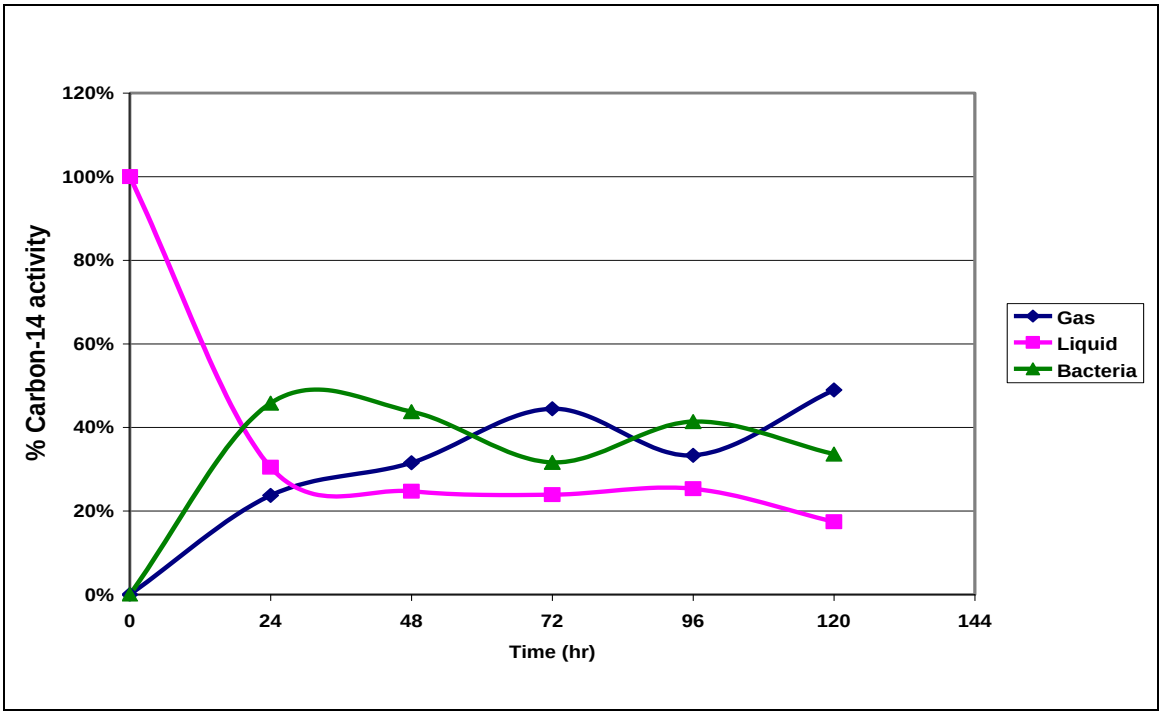



Figure 13: Activity distribution in bioreactors containing $^{14}C$-labeled $NaC_2H_3O_2$ as a carbon source.

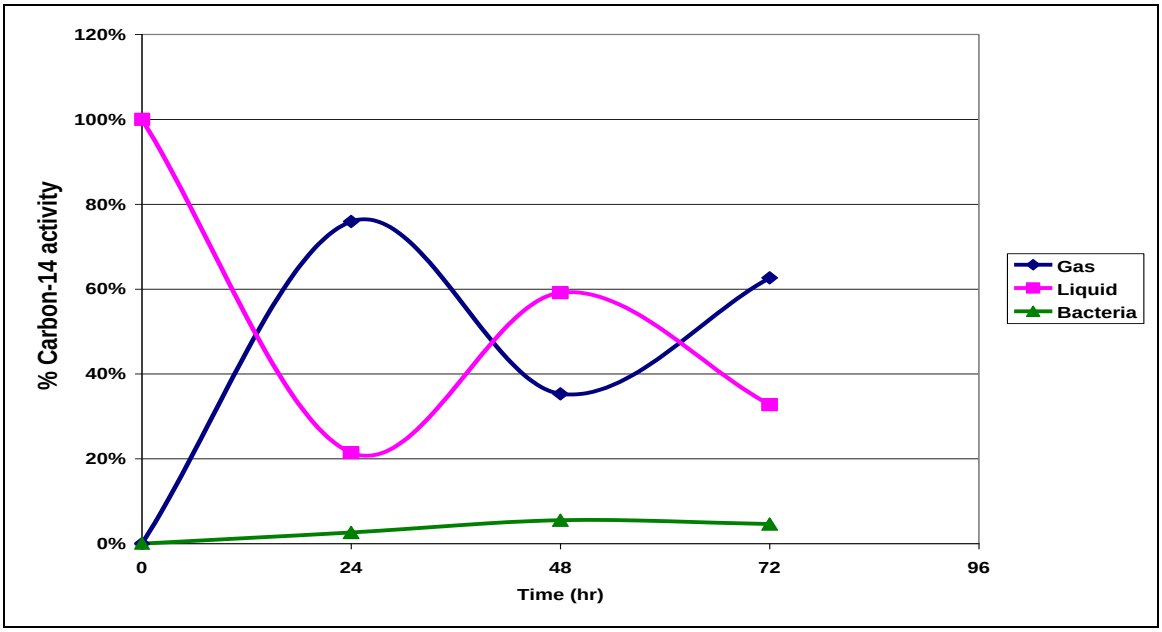



Figure 14: Activity distribution in bioreactors containing $^{14}C$-labeled $NaHCO_3$ as a carbon source.

At time zero in the sodium acetate bioreactor system, (Figure 14) all the activity was contained in the liquid phase because the $^{14}C$-labelled $NaC_2H_3O_2$ salt was dissolved in the growth

medium. The bacteria consumed the $NaC_2H_3O_2$ 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 $^{14}C$ 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 1) when heated.

$$H_20 + 2NaC_2H_3O_2 \rightarrow Na_2CO_3 + 2CH_4 + CO_2 \quad (1)$$

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 $^{14}C$-labelled $NaHCO_3$, all the activity was contained in the liquid phase, as was the case for $NaC_2H_3O_2$. 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 $NaHCO_3$ chemical decay to $CO_2$ (equation 2).

$$2NaHCO_3 \rightarrow Na_2CO_3 + H_20 + CO_2 \quad (2)$$

Results from experiments conducted with $^{14}C$-labelled sodium salts but no bacteria offer further evidence that bacteria consume the salts. With no bacteria present, ~69% of the activity in the $NaC_2H_3O_2$ experiments stayed in liquid phase at 24 hours, with the balance going to the gas phase. In contrast, the liquid phase of the $NaC_2H_3O_2$ system charged with bacteria contained ~30% of the $^{14}C$ activity at 24 hours. In the $^{14}C$-labelled $NaHCO_3$ system without bacteria, ~75% of the $^{14}C$ activity was detected in the gas phase with the remainder in the liquid. This result further supports the theory of $NaHCO_3$ decomposition due to chemical instability.

.

## Conclusions

Results of experiments described in this paper indicate microbial remediation may be a suitable treatment option for irradiated graphite from nuclear reactors. The characterized microbe mixture was able to grow in the presence of, and on, inert graphite. Furthermore, the microbes processed $^{14}C$ target species present in a chemical form thought to be similar to that of $^{14}C$ compounds expected to form in irradiated graphite. Experiments with, and analysis of, irradiated graphite are necessary to determine the chemical form of $^{14}C$ and the viability of bacterial processing of that chemical in the radioactive environment. The actual chemical form(s) of $^{14}C$ in irradiated graphite may be a determining factor in the ultimate feasibility of such an application.

# References

Assih, E.A., Ouattara, A.S., Theirry, S., Cayol, J-L., Labat, M., Macarie, H., Stenotrophomonas acidaminiphila sp. Nov., strictly aerobic bacterium isolated from an upflow anaerobic sludge blanket (UASB) reactor.*Internatinal Journal of Systematic and Evolutionary Microbiology*, 52: 559-568 (2002)

Aviam, O., Bar-Nes, G., Zeiri, Y., Sivani, A., Accelerated Biodegradation of Cement by Sulfur-Oxidizing Bacteria as a Bioassay for Evaluating Immobilization of Low-Level Radioactive Waste. *Applied and Environmental Microbiology*, 70 (10): 6031-6036 (2004)

Battista, J.R., Against All Odds: The Survival Strategies of Deinococcus radiodurans. Annual Review of Microbiology, (51), 203-224 (1997)

Boonchan, S., Britz, M.L., Stanley, G.A., Surfactant-enhanced biodegradation of high molecular weight polycyclic aromatic hydrocarbon by *Stenotrophomonas maltophilia. Biotechnology and Bioengineering*, 59 (4): 482-494 (1998)

Brierley, J.A., Kulpa, C.F.Jr., Biometallurgical treatment of precious metal ores having refractory carbon content. United States Patent 5244493 (1993)

Chicote, E., Moreno, D., Garcia, A., Sarro, I., Lorenzo, P., and Moretero, F., Biofouling on the walls of a spent nuclear fuel pool with radioactive ultra pure water. Biofouling 20: 35-42 (2004)

Dadachova E., Bryan R.A., Huang X., Moadel T., Schweitzer A.D., Aisen P., Nosanchuk J.D., Casadevall, A., Ionizing Radiation Changes the Electronic Properties of Melanin and Enhances the Growth of Melanized Fungi. *PLoS One*, May 23 (2007)

Dunzik-Gougar, M.L., van Raavensway, F., Kuczynski, L., and Slabber, J.M.S. Microbial treatment of irradiated graphite for separation of radioisotopes $^{14}C$ from bulk graphite $^{12}C$. Papers read at the 4th topical meeting on High temperature Reactor Technology held in Washington, D.C., USA from September 28 to October 1 (2008)
Electric Power Research Institute (EPRI), Graphite decommissioning: Option for Graphite Treatment, Recycling, or Disposal, including a discussion of Safety-Related Issues, Doc no. 1013091, D. Bradbury and A. Wickham, Principal Investigators (2006)

Emerson, D. and Moyer, C., Isolation and characterization of novel iron-oxidizing bacteria that grow at circumnetral pH. *Applied and Environmental Microbiology*, 63 (12): 4784-4792 (1997)

Fachinger, J. “Decontamination of Nuclear Graphite,” 3rd topical meeting on High temperature Reactor Technology held in Gautang, South Africa, October (2006)

Fachinger, J., Podruhzina, T., von Lensa, W., “Decontamination of Nuclear Graphite by Thermal Treatment,” Solutions for Graphite Waste, Manchester, UK, March (2007)

Gleeson, DB., Kennedy, N.M., Clipson, N., Melville, K., Gadd, G.M., McDermott, P., Characterization of bacterial community structure on a weathered pegmatitic granite. *Microbial Ecoloby*, 51: 526-534 (2006)

IAEA (International Atomic Energy Agency), Characterization, Treatment and Conditioning of Radioactive Graphite from Decommissioning of Nuclear Reactors, IAEA-TECDOC-1521, Waste Technology Section, IAEA, Vienna (2006)

Kim, C.H., Han, S. H., Kim, K.Y., Cho, B.H., Kim, Y.H., Koo, B.S., Kim, Y.C., Cloning and expression of pyrroloquinoline quinone (PQQ) genes from a phosphate solubilising bacterium *Enterobacter intermedium. Current Microbiology*, 47: 457-461 (2003)

KhrIpunov, V. I., Kurbatov, D. K, and Subbotin, M. L., Carbon-14 production in CTR material and blankets. Journal of Fusion Energy, Volume 27, Number 4, pp 241-249, December (2008)

LiPuma,J.J., Spilkeer, T., Coenye, T., LiPuma, C.F.G.J.J., Spilker, T., Coenye, T., Gonzalez, C.F., An epidemic Burholderia cepacia complex strain identified in soil. *The Lancet*, 359 (9322): 2002-20030 (2002)

Lloyd, J.R., Microbial reduction of metals and radionuclides. FEMS Microbiology reviews 27, 411-425. 1 (2003)

Marsden, B.J., Hopkinsin, K.L., Wickham, A.J., "The Chemical Form of Carbon-14 in Irradiated Graphite," Nirex Limited, Issue 4 pp. 8-11, United Kingdom (2002)

Mason, J.B., and Bradbury, D. 1992. Pyrolysis and its potential use in nuclear graphite disposal. Proceedings of Technical committee meeting on nuclear great graphite waste management held in Manchester, UK, on the 18 to 20 October (1992)

Nicomrata, D., Dicka, W.A., Dopsonc, M., Tuovinen, O.H., Bacterial phylogenetic diversity in a constructed wetland system treating acid coal mine drainage. *Soil Biology & Biochemistry*, 40: 312-321 (2008)

Nies, D.H., Microbial heavy metal resistance. *Applied Microbiology and Biotechnology*,51: 730-750 (2008)

Sembries, S. and Crawford, R.L., Production of *Clostridium bifermentas* spores as inoculum for bioremediation of nitroaromatic contaminats. *Applied and Environmental Microbiology*,63 (5): 210-214 (1997)

Sing, R., Gupta, P., Purohit, S., Kumar, P., Vaijapurkar, S.G., Chackarkar, Radiation resisance of the microflora associated with amniotic membranes. *World Journal of Microbiology and Biotechnology*, 22: 23-27 (2006)

Springael, D., Diels, L., Hooyberghs, L., Kreps, S., Mergeay, M, Construction and characterization of heavy metal resistant haloaromatic-degrading *Alcaligenes eutrophus strains*. *Applied and Environmental Microbiology*, 59: 334-339 (2003)

tyriaková, I., Bhatti T.M., Bigham, J.M., Styriak, I., Vuorinen, A., Tuovinen, O.H., Weathering of phlogopite by Bacillus cereus and Acidthiobacillus ferroxidans. Canadian journal of Microbiology, 50: 213-219 (2004)

Suresh, K., Prakash, D., Rastogi, N., Jain, R.K., *Clostridium nitrophenolicum* sp. Nov., a novel anaerobic p-nitrophenol-degrading bacterium, isolated from a subsurface soil sample. *International Journal of Systematic and Evolutionary Microbiology*, 57: 1886-1890 (2007)

Takahashi, R., Toyahara, M., Maruki, S. and Ueda, H., Investigation of morphology and impurity of nuclear grade graphite, and leaching mechanism of Carbon-14: Proceedings of Technical committee meeting on nuclear great graphite waste management held in Manchester, UK, on the 18 to 20 October (1992)

Takahashi, R., Toyahara, M., Maruki, S., Ueda, H., Yamamoto, H., Investigation of morphology and impurity of nuclear- Grade graphite, and leaching mechanism of carbon-14, 176, IAEA Technical Committee Meeting on “Nuclear Graphite Waste Management“, United Kingdom (2004)

Valverde, A., Delvasto, P., Peix, A., Velázquez, Santa-Regina, I., Ballester, A., Rodrĭgiez-Barrueco, C., Garcĭa-Balboa,C., Igual, J.M., *Burkholderia ferrariae* sp. Nov. isolated from an iron ore in Brazil. *International Journal of Systematic and Evolutionary Microbiology*, 56: 2421-2425 (2006)

von Lensa, Werner, presentation at 7th EPRI International Decommissioning and Radioactive Waste Workshop (in collaboration with EDF-CIDEN), Lyon, France (2008)

Wentzel, A., Elligensen, T.E., Kotlar, H-K., Zotchem, S.B., Throne-Holst, M., Bacterial metabolism of long-chain n-alkanes.*Applied Microbiology and Biotechnology*.76: 1209-1221 (2007)

Wjote, O., Eisen, J.A, Heidelberg, J.F., Hickey, E.K., Peterson, J.D., Dodson, R.J., Haft, D.H., Gwinn, M.L., Nelson, W.C., Richardson, D.L., Moffat, K.S., Qin, H., Jiang L., Pamphile, W., Crosby, M., Shen, M., Vamathevan, J.J., Lam, P., McDonald, L., Utterback, T., Zalewski, C., Makarova, K.S., Aravind, L, Daly, M.J, Minton, K.W., Fleischmann, R.D., Ketchum, K.A., Nelson, K.E., Salsberg, S., Smith, H.O., Venter, J.C., and Fraser C.M., Genome sequence of the radio resistant bacterium Deinococcus radiodurans R1. Science, 286 (5444), 1571-7 (1999)

Williams, P.J., Pretorius, R., Bapela, I.I. Pete, G.A., Phihlela, L.C., Mngadi, N.S., Dunzik-Gougar M.L., “Viability study for the microbial treatment of radioactive graphite waste contaminated with $^{14}C$,” Journal of Biotechnology, Volume 150, Supplement 1, Page 245, November (2010)

Yoshida, N., Yagi, K., Sato, D., Watanabe, N., Kuroishi, T., Nishimoto, K., Yanagida, A., Katsuragi, T., Kanagawa, T., Kurane, R., Tani, Y., Bacterial communities in petroleum oil in stockpiles, *Journal of Bioscience and Bioengineering* 99(2): 143-149 (2005)