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Biodegradation of Liquid Scintillant Cocktails

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ABSTRACT
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BIODEGRADATION OF LIQUID SCINTILLANT COCKTAILS

by Anneke Twaambo Lubben

Liquid scintillant cocktails (LS cocktails) are widely used by both research establishments and various industries in the quantification of radionuclides. Such cocktails contain a mixture of organic compounds including solvents, primary and secondary fluors and surfactants. Current disposal methods include sewer discharge and incineration. However, increased environmental pressures and the introduction of new legislation aiming to curb emissions from incinerator facilities necessitate the investigation of alternative disposal methods. This study considers the use of biodegradation as an alternative means of waste disposal for two common liquid scintillant cocktails, EcoSafe™ and Gold Star™.

Sequencing batch reactors were used containing activated sludge (AS) as a biomass source, a standard nutrient medium and a carbon source in the form of a LS cocktail or one of its components. Compounds of interest were butoxyethoxy ethanol (BEE), di-isopropyl naphthalene (DIPN), branched alkyl benzenes (BAB), 2,5-diphenyl oxazole (PPO), bis-(*o*-methylstyryl) benzene (bis-MSB) and nonylphenol ethoxylates (NPEs). All experiments were conducted under laboratory conditions with no temperature, pressure or light controls. Aeration was used to ensure aerobic conditions and mixing. Parameters monitored in key bioreactors included temperature, aeration rate and pH. Samples were analysed for biological oxygen demand (BOD_5), microbial densities and compound concentrations. Microbiological assessments were agar plating enumerations and optical density measurements using spectrophotometry. Compound concentrations were monitored by UV-fluorescence spectrometry and gas chromatography-mass spectrometry (GC-MS).

pH data show that reproducible patterns of change in hydrogen ion concentrations were occurring in the bioreactors. The lag phase prior to the onset of these fluctuations were reduced by the use of a pre-adapted biomass. Pre-adaptation not only reduces lag times but also allows for the survival of a viable biomass at elevated liquid scintillant cocktail concentrations, up to 40 % loading by volume.

Activated sludge was shown to be a suitable microbial source for biodegradation experiments, with morphological classifications following agar plating showing a presence of 14 different microbial species. Natural selection to the different compounds of interest was also shown by a reduction in species numbers. The microbiological techniques were used to determine the toxicity and substrate utilisation for growth of the compounds of interest. Neither of the LS cocktails, nor any of its components showed a toxic effect on the biomass. A link was evident between biomass growth, as assessed by the microbiological techniques and compound losses as determined by GC-MS.

Due to analytical limitations compound concentrations were only determined for BEE, DIPN, BAB and PPO. Bis-MSB and NPEs were not assessed. Both biotic and abiotic losses are quantified at a compound concentration level equal to that in a 1 % LS cocktail solution. Losses were found for BEE and PPO. BEE losses were primarily attributable to biomass increases and therefore biodegradation. Abiotic losses dominated for PPO. DIPN showed no biotic or abiotic losses under the conditions used. This contradicts the outcomes from other studies which showed that biodegradation did occur, albeit at much lower levels (0.005 %). The sixteen BAB isomers are grouped according to the length of their shorter alkyl chain. It was evident that selective losses were occurring as a function of this chain length. Those with one or two atoms in their aliphatic chain showed large biotic and abiotic losses. With increased chain length compound losses were reduced, particularly regarding biotic losses. Degradation rates were calculated by first order rate equations. Both lag time and degradation rate were directly related to compound recalcitrance. The experimental technique did not allow for the detection of degradation products. Despite limited control of degradation conditions, and the use of fresh activated sludge for each experiment, the extent of compound loss was shown to be reproducible.

A commercial viability investigation and the basis for an environmental impact assessment were included in this study.

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And now it is back to 'waking up and smelling the roses'
followed by a bit of 'dancing in the moonlight' .....

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This PhD dissertation by

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has been produced under the supervision of the following persons

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Mr. Trevor Clayton

"If you always do what you've always done,
you'll always get what you've always got"

Anon.

Chapter 1 - Introduction

Liquid scintillation counting dates back to the early 20th Century. It is a well established and sensitive method used by research establishments and the nuclear, medical, pharmaceutical and biochemical industries for the measurement of radioactivity. Scintillation counting works on the principle of a transfer of ionisation and/or excitation energy from the radionuclides in an aqueous sample to produce light photons that are detected by a liquid scintillation counter. The number of light pulses detected is directly proportional to the radioactive content of the sample. Details of this process are given in Appendix 1. The measurement is enabled by mixing the sample to be analysed with a suite of organic chemicals, known as a liquid scintillant cocktail. It is this chemical mixture that is the focus of this study.

1.1 Liquid scintillant cocktails (LS cocktails)

Liquid scintillant cocktails contain various components that are necessary for the energy transfer from radiation to light energy for counting. Primary and secondary scintillators, primary and co-solvents, and emulsifiers are all included in the mixture.

Emulsifiers, like nonylphenol ethoxylates (NPEs), are used to ensure thorough mixing of the aqueous sample with the organic components of the liquid scintillant cocktail. The co-solvent is present to dissolve the primary solvent, fluors and radiation source. Methoxy ethanol and ethoxy ethanol are often used for this purpose. Di-isopropyl naphthalene (DIPN) is used as a primary solvent. Its purpose is to convert kinetic energy from the radionuclides into excitation energy. The primary scintillator has the role of absorbing excitation energy from the primary solvent and emitting it as fluorescence. A commonly used example is 2,5-diphenyl oxazole (PPO) which is readily activated, and has a maximum fluorescence at 3650 nm (Kobayashi & Maudsley, 1974). The role of the secondary fluor is to absorb this emitted fluorescence and re-emit it at a higher wavelength. This also acts to amplify the light output from the sample. Bis (*o*-methylstryl) benzene (bis-MSB) is a common example and has a maximum fluorescence at 4160 nm (Kobayashi & Maudsley, 1974).

Historically, LS cocktails have been prepared with benzene, *p*-dioxane, toluene or xylene as the organic solvent base. The major disadvantage of these solvents is the low flash point, the temperature at which they volatilise (4-10 °C). This not only results in substantial and variable solvent losses, affecting the counting reproducibility of the liquid scintillant cocktail, but also has fire and health implications. Around thirty years ago a different LS cocktail composition started to be used. This used solvents with higher flash points, such as 1,2,4-trimethylbenzene with a flash point of 47 °C. Despite the benefits of improved counting reproducibility and reduced flammability, this alternative resulted in a toxicity increase of the cocktail (Klein & Gershey, 1990; Hawkins, 1991). Further developments have resulted in the use of solvents, such as di-isopropyl naphthalene, that consist of long chained, multi-ringed aromatic groups. These have both increased flash points and reduced toxicity, and are generally considered to be less hazardous (Passo & Cook, 1994). They are therefore not treated as hazardous waste (Klein & Gershey, 1990), and in some cases LS cocktails based on these solvents are being marketed as biodegradable or environmentally safe.

1.2 Disposal options for liquid scintillant cocktails

Following analysis of the sample – liquid scintillant cocktail mixture by liquid scintillant counting, the liquid and holding vessels are disposed of. Approximately 80,000 litres annually of liquid scintillant cocktail is produced and sold in the United Kingdom alone (Smythe, 2001, *pers. comm.*). As the aqueous sample cannot be separated from the LS cocktail reuse is not an option. The two fractions of the waste that cause particular concern are the radioactive and organic components.

The disposal method required for radioactive waste is primarily determined by a class system, where the waste is grouped according to the nature and quantity of radioactivity associated with it. LS cocktail waste is generally classed as low level waste (LLW). For liquid LLW it is widely accepted that the most practical method for disposal is dilution in the wastewater system. Dilution factors here are usually sufficient to reduce the activity below the levels deemed to be hazardous to health (Bovard & Candillon, 1960; Klein & Gershey, 1990).

Disposal into the sewer system would also result in dilution of the organic fractions, however, their subsequent biodegradation cannot be assured.

With an increase in public awareness of environmental issues and legislation like the EC Directive on the Control of Dangerous Substances to the Aquatic Environment (76/464/EEC) minimising disposal of pollutants into the environment, the dilution option is becoming less acceptable. In addition, the development of a 'polluter-pays' principle for the UK water industry, whereby a charge is made for the load of toxic chemicals discharged into the sewers, has resulted in increased disposal costs.

Although, in the United Kingdom, limited sewer discharge of liquid scintillant cocktails does occur under authorisation, the majority of the waste is disposed of by incineration. This disposal option also has its shortcomings as combustion can result in significant atmospheric pollution, including the release of dioxins and particulates. In addition, the implementation of the Climate Change Levy in April 2001, aiming to reduce the production of incineration gases, will result in further price increases.

1.3 This study

It is evident that a disposal method that overcomes the limitations involved in sewer discharge and incineration has to be established for liquid scintillant cocktail waste. This project therefore investigates harnessing the metabolic capabilities of microorganisms to break down organic compounds. This is termed biodegradation and has been extensively utilised in organic pollutant remediation.

Initial experiments would need to be conducted using laboratory-scale bioreactors. For the developed process to be used on a commercial basis, scaling up to a commercially viable volume would need to be achieved, along with attracting a market share of the LS cocktail waste disposal market.

To assess the use of biodegradation as a viable waste disposal option for liquid scintillant cocktail waste, this study aimed to:

- determine the biodegradation potential of commercially supplied liquid scintillant cocktails as a precursor to further waste disposal into a sewer system,
- establish optimum conditions for degradation using activated sludge,
- identify the process degradation products and their environmental impacts, and
- investigate scale-up issues accompanied by a commercial viability study and environmental impact assessment.

Two commercially supplied liquid scintillant cocktails were used, namely Gold Star™ and EcoSafe™. These contain di-isopropyl naphthalene (DIPN) and branched alkyl benzenes (BAB) respectively as their primary solvent (Table 1.1). The composition of these cocktails includes the basic requirements of any liquid scintillant cocktail, and is comparable to that of other cocktails, where only relative compositions and solvent type used tends to vary.

Table 1.1 Essential components of the two liquid scintillant cocktails used and their roles

Role	*Compound name*	*Acronym*	*Composition (% by volume)*	*LS cocktail present in*
Primary scintillator/fluor	2,5-diphenyl oxazole	PPO	~1 %	Gold Star™ and EcoSafe™
Secondary scintillator/fluor	Bis-(*o*-methylstyryl) benzene	bis-MSB	~1 %	Gold Star™ and EcoSafe™
Primary solvent	Di-isopropyl naphthalene Branched alkyl benzenes	DIPN BAB	~75 % with BEE ~75 %	Gold Star™ EcoSafe™
Co-solvent	Butoxyethoxy ethanol	BEE	~75 % with DIPN	Gold Star™
Emulsifier	Nonylphenol ethoxylate	NPE	~23 %	Gold Star™ and EcoSafe™

Chapter 2 - Literature review

Waste disposal and managed disposal pathways are not a recent development in our society. However it is only in the last fifty years that environmental issues have come to the fore with the realisation that our present use of resources is not sustainable.

In the United States, the Toxics Release Inventory (TRI) has been set up listing the annual emissions to air, water and land of over 600 chemicals and chemical categories designated as toxic by the Environmental Protection Agency (EPA). In addition, over 100 organic compounds have been assigned the status of priority pollutant by the EPA (Mohammed *et al.*, 1996). The European Union and the Environment Agency (EA) of the United Kingdom have established similar databases into which several of the chemicals present in liquid scintillant cocktails are included. The main problem associated with the assessment of environmental impacts of chemicals is the lack of data on mechanisms resulting from their introduction. Most of the processes involved will result in the dispersal and dilution of chemicals, but some will act to concentrate them (Roberts, 1998). Often studies are carried out in the laboratory, but these data can not necessarily be extrapolated to the natural system, and usually only involves single compounds, thus neglecting to take cumulative or synergistic effects into account (Jones & de Voogt, 1999; Lye *et al.*, 1999; Templeton, 1976).

For compounds to display toxic properties, they must be bioavailable (Henner *et al.*, 1997), with the substance concentrating in single or multiple target organs or tissues (Mackay & Fraser, 2000). It is widely accepted that organisms can achieve high concentrations of organic contaminants relative to levels found in their immediate environment. In this context 'environment' includes the habitat they reside in, and the air and food they respire and consume. This concentration phenomenon is referred to as bioaccumulation.

Organisms that bioaccumulate pollutants can be used as indicators and monitors of pollution. Concentrations found in the organism then represent a time-integrated picture of concentrations in the ambient environment (Næs *et al.*, 1998), with levels depending on the proximity of the pollutant source and the exposure time. Concerns arise when bioaccumulation occurs to such an extent

that toxic responses in the receiving organism become apparent. Toxicity may vary with factors other than the pollutant chemistry, such as the sex, health, age, and reproductive and nutritional status of the organism. It is therefore important that all comparative toxicity tests are carried out under identical conditions and with the same species. Unfortunately this is rarely the case (Addison, 1983).

In addition to the toxicity to organisms, the pollutant may be metabolically resistant and therefore be conserved as it passes from organism to organism in the food chain. Potentially this results in cumulatively increasing concentrations of toxins (Hutchins *et al.*, 1998; Mackay & Fraser, 2000), therefore having implications for public health (Choori *et al.*, 1998; Jones & de Voogt, 1999; Lee, 1977). Persistent organic pollutants are typically hydrophobic and lypophilic, hence they accumulate in fatty material, and avoid the aqueous phase (Jones & de Voogt, 1999). It is as a result of this partitioning behaviour that they readily concentrate in stable tissue such as those with lipid stores (Lee, 1977).

Marine species are known to be very effective bioaccumulators. Bivalves are capable of concentrating hydrocarbons several orders of magnitude over concentrations in the water (Fairey *et al.*, 1997; Hutchins *et al.*, 1998). Petroleum hydrocarbon exposed mussels from Kiel Fjord showed an aromatic hydrocarbon concentration factor of up to 10,000 times (Lee, 1977). In fish it has been found that only alkanes longer than C_{28} were retained in the tissue whilst shorter alkanes were discharged via the urine and faeces (Lee, 1977). The accumulation of oil in these commercial species contributes to the transfer of hydrocarbons through the food chain and eventually to man. The potential for bioaccumulation does not only apply to hydrocarbons but also to many other environmental pollutants, including other organic compounds, metal ions and radionuclides.

As for general organic waste management (Sonesson *et al.*, 2000), liquid scintillant cocktails have historically been disposed of in one of two ways, initially by dilution into the sewer system and more recently primarily by incineration. This chapter aims to give an overview of these two disposal

mechanisms, their impacts on the environment and the possible alternative of bioremediation as is being investigated in this research. A case study of problems found with the incineration of liquid scintillant cocktails at Grundon plc., Slough is included.

2.1 Environmental effects of liquid scintillant cocktails if disposed of by dilution

Despite sewer disposals usually passing into the marine environment via a wastewater treatment plant, a proportion of the contaminants are not removed by this process and therefore pass into the aquatic environment (Nyholm, 1992). Industrial waste water can contain a variety of dissolved inorganic and organic compounds and heavy metals which may be toxic even at trace levels (Benner *et al.*, 1998; Choori *et al.*, 1998; Nyholm, 1992).

2.1.1 Solvents and scintillators

Industrial effluents often contain solvents that are difficult to remove from the effluent solution and therefore not only have a toxic effect on biomass in the wastewater treatment works but also readily contaminate rivers, lakes and sediments (Singleton, 1994).

2.1.1.1 Butoxyethoxy ethanol (BEE)

Butoxyethoxy ethanol (Figure 2.1) is an organic solvent that has many industrial applications. It is known under a variety of names including:

- 2-(2-butoxyethoxy)ethanol
- diethylene glycol *n*-butyl ether
- 2-ß-butoxyethoxy ethanol
- butoxydiethylene glycol
- butoxydoglycol
- butyl diglycol
- *o*-butyl diethylene glycol
- diglycol monobutylether, and
- butyl diethyl cellosolve.

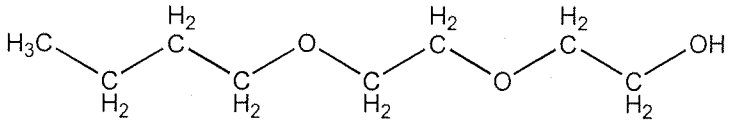


Figure 2.1 Structure of butoxyethoxy ethanol (BEE)

Despite its extensive use in industry little is known about its metabolic fate in animals and man (Thijssen *et al.*, 1986). BEE has not been classified as biodegradable.

2.1.1.2. Di-ispropyl naphthalene (DIPN)

Di-isopropyl naphthalene (DIPN) is one of the solvents present in many liquid scintillant cocktails, including Gold Star™. It is a clear, colourless and odourless liquid with a molecular weight of 212 Da and a boiling range of 290–299 °C, which is readily adsorbed onto particulate matter (Addison, 1983). When used in industry it often contains a mixture of isomers, the main components of which are 2,6- and 2,7- alkylated isomers (Höke & Zellerhoff, 1998). DIPN is extensively used as a solvent in the manufacture of carbon-less copy paper (Addison, 1983; Höke & Zellerhoff, 1998) and therefore its toxicological properties have been widely studied. It has a very high flash point (152 °C) and as such is classified as non-dangerous in accordance with national and international traffic regulations.

DIPN (Figure 2.2) is a polycyclic aromatic hydrocarbon (PAH), and as such has some of the toxicity properties of this group of compounds. PAH are hydrophobic organic compounds, consisting solely of hydrogen and carbon atoms and containing two or more linked benzene rings (Bennett *et al.*, 1999; Brubaker & Stroo, 1992; O'Neill, 1993). Due to numerous natural and anthropogenic sources, PAH are ubiquitous in the environment (Cerniglia, 1984; Shiaris, 1989; Wilson & Jones, 1993). They have a tendency to accumulate in soils and tissues as a result of their relatively stable, recalcitrant and biodegradation-resistant natures. Some family members are also known to have mutagenic and carcinogenic properties (Ahn *et al.*, 1999; Boonchan *et al.*, 1998; Pothuluri & Cerniglia, 1994; Shuttleworth & Cerniglia, 1995; Warith *et al.*, 1999;

Wilson & Jones, 1993; Woodhead *et al.*, 1999; Yang *et al.*, 1999), and thus cause concern as environmental pollutants when present in significant concentrations.

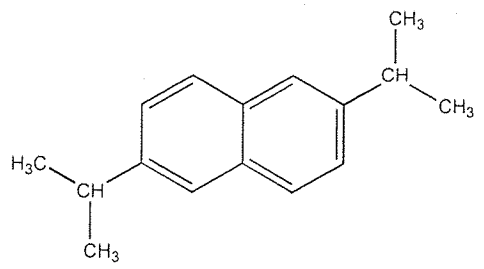


Figure 2.2 Structure of 2,6-di-isopropyl naphthalene (DIPN)

Naphthalene compounds and many of their derivatives have been designated as priority pollutants by the EPA in the United States (Warith *et al.*, 1999; Wilson & Jones, 1993). Most of them are toxic or inhibitory to specific biological pathways, and therefore pose a problem when disposed of into the aquatic environment (Zhu *et al.*, 1999). DIPN however is considered as biodegradable (Klein & Gershey, 1990; Passo & Cook, 1994) and has a short environmental half-life and low bioaccumulation potential (Addison, 1983; Sumino, 1977). A study carried out by Osaki and Ikeda (1976, cited in Addison, 1983) indicated that when high doses of DIPN isomers were administered to fish, effects on their serum enzymes were detected, suggesting liver and kidney damage. Yoshida & Kojima (1978a) reported a two hundred-fold bioaccumulation factor of DIPN in carp and grey mullet, being detected in the gallbladder, hepatopancreas, and adipose tissue within 24 hours of introduction. The biological half-life of the radiolabelled DIPN used was found to be 56 hours (Kojima *et al.*, as cited in Klein & Gershey, 1990).

2.1.1.3 Branched alkyl benzenes (BAB)

The solvent used in EcoSafe™ consists of branched alkyl benzenes. BAB contain a single aromatic ring with a branched alkyl chain (Figure 2.3). In the case of the forms present in EcoSafe™, the alkyl chain is attached to the benzene ring at position one. The alkyl chains vary in length even within a family of the same molecular weight. BAB are used extensively in liquid

scintillant cocktails, often being referred to as linear alkyl benzenes, and are considered to be biodegradable (Klein & Gershey, 1990).

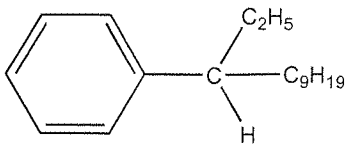


Figure 2.3 Structure of a $C_{18}H_{30}$ branched alkyl benzene (BAB) isomer

The toxicity of BAB has not been extensively studied, although it is generally accepted that either branching or a shorter alkyl chain on the benzene ring will increase toxicity (Klein & Gershey, 1990). Linear alkyl benzenes with an alkyl chain length of between C_{10} and C_{13} are included in the first priority list of the European Union (EU), although this classification remains controversial. The list has been established in the application of EU Council Regulation 93/793 concerning the Assessment and Control of Risks presented by Existing Chemicals (Binetti *et al.*, 2000).

Although these specific isomers are not included in EcoSafe™, alkyl chain lengths up to and including C_{10} are present. A study by Warner & Kimerle (1982, as cited in Binetti *et al.*, 2000) showed a concentration factor of 35 for C_{12}- alkylbenzene in the fish species *Lepomis macrochirus*. This is considered to be a low bioconcentration level and may be explained by the high metabolic rate of the substance in fish. No further laboratory studies have been carried out into the toxic effects of branched alkyl benzenes, but various models have been used to calculate the following bioconcentration factors:

- earthworms - 326
- meat - 0.08
- milk - 0.03 (Binetti *et al.*, 2000)

2.1.1.4 2,5-diphenyl oxazole (PPO) and bis-methylstyryl benzene (bis-MSB)

PPO (Figure 2.4) and bis-MSB (Figure 2.5) are used in liquid scintillant cocktails as fluors, necessary to convert excitation energy into light energy for quantification.

No literature has been found on the environmental impacts of these compounds, however they have not been certified as biodegradable in the available hazard data sheets, and therefore pose a problem when disposal options are considered.

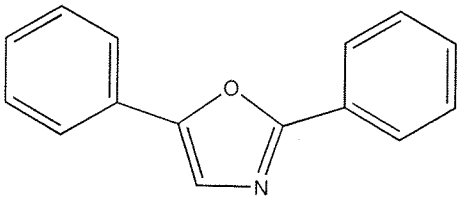


Figure 2.4 Structure of 2,5-diphenyl oxazole (PPO)

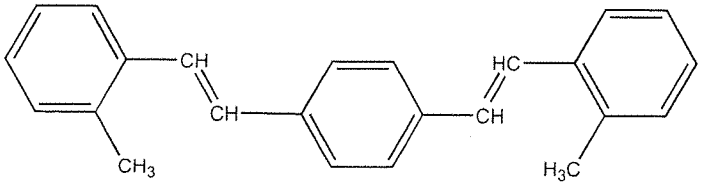


Figure 2.5 Structure of 1,4-bis (2-methylstyryl)benzene (bis-MSB)

2.1.2 Nonylphenol ethoxylate (NPE) surfactants

Surfactants are surface-active substances that alter the surface tension of liquids, greatly enhancing the solubility of a compound in solution. They consist of a hydrophilic polar head and a hydrophobic non-polar tail (Abd-Allah & Srorr, 1998; Rouse *et al.*, 1994), and accumulate at gas/liquid, liquid/liquid and solid/liquid interfaces (van Gunkel, 1996). Various classifications are used for surfactants. These include using their source (synthetic and biological surfactants) and the nature of the charge associated with the head of the molecule (non-ionic, anionic, cationic and zwitterionic or amphoteric) (Page *et al.*, 1999; Rouse *et al.*, 1994; Scullion *et al.*, 1996). Surfactants are a classic example of persistent organic pollutants, being both hydrophobic and lypophilic. It is worth noting that biological surfactants (also known as biosurfactants) show

low cellular toxicity and are considered to be biodegradable (Singer, 1985, as cited in Page *et al.*, 1999).

Nonylphenol ethoxylates (NPEs) (Figure 2.6) are non-ionic surfactants that have been employed for decades in formulations used in domestic laundry products, textile manufacture, industrial cleaning, pulp and paper production and as a dispersant for pesticide solutions (Bennie *et al.*, 1997; Blackburn *et al.*, 1999; Evans *et al.*, 1997; Furlong & Gates, 1997; Plomley & Crozier, 1998; Shang *et al.*, 1998, 1999a, 1999b). They possess the properties of good wetting, detergency, low foaming and low cost (Bennie *et al.*, 1997). They are typically used as a mixture of isomers of different ethoxylate chain lengths, ranging from one to twenty ethoxy units with the largest abundance of nine and ten units (Argese *et al.*, 1994; Crescenzi *et al.*, 1995b; Shang *et al.*, 1999a). They are one of the most widely used classes of non-ionic surfactants worldwide (Naylor, 1995; Scarlett *et al.*, 1994). In the United Kingdom alone 18,000 tonnes of NPE are produced annually (Blackburn *et al.*, 1999; Lye *et al.*, 1999), with 50 % being used for industrial and institutional cleaning products that are subsequently disposed of into the sewer system and eventually pass into the aquatic environment (Lye *et al.*, 1999; Mann & Boddy, 2000). Despite synthetic surfactants employed in the consumer market being thoroughly environmentally tested, these same extensive tests are not mandatory for industrial use (Page *et al.*, 1999).

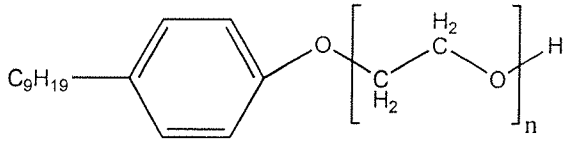


Figure 2.6 Structure of nonylphenol ethoxylate (NPE) isomers

There are concerns over the disposal of synthetic surfactants into the aquatic environment due to their persistence, low biodegradability and toxicity (Abd-Allah & Srorr, 1998; Sherrard *et al.*, 1996; Rycroft *et al.*, 1994; Schröder *et al.*, 1999). Surfactants are known to affect living cells by impairing the functioning and integrity of biological membranes (Florence *et al.*, 1984, as cited in

Shcherbakova *et al.*, 1999). Due to their lipophilicity, they also have a tendency to accumulate in the tissues of some organisms (Ejlertsson *et al.*, 1999). Staples and co-workers (1998, as cited in Beeson *et al.*, 1999) found a bio-concentration factor of up to 250 and 487 for octyl- and nonyl- phenol ethoxylates in laboratory studies and field studies respectively. In rainbow trout changes in body weight, fork length and the ovosomatic index were apparent following exposure to alkylphenol ethoxylates from hatch day (Ashfield *et al.*, 1998, as cited in Beeson *et al.*, 1999). Surfactants can be toxic to bacteria, algae, daphnids and fish at concentrations as low as 1 mg/L (Lewis, 1990, as cited in van Gunkel, 1996; Schröder, 1993).

The biodegradation of NPEs occurs exclusively at the polyethoxylate chain resulting in a shortened chain, with the hydrophobic aromatic group being left intact (Maki *et al.*, 1996, 1998). With a decreased ethoxylate chain length there is not only a corresponding increase in aquatic toxicity of the resultant alkylphenols, but also an increase in lipophilicity and hence the potential for bioaccumulation in some organisms (Bennie *et al.*, 1997; Sherrard *et al.*, 1996; Dorn *et al.*, 1993; Field & Reed, 1999; Kibbey *et al.*, 1996; Lye *et al.*, 1999; Maki *et al.*, 1996, 1998; Plomley & Crozier, 1998; Plomley *et al.*, 1999; Scarlett *et al.*, 1994; Shang *et al.*, 1998; Sherrard *et al.*, 1996). Non-ionic surfactants are considered to be less toxic to microorganisms than cationic or anionic forms (Boonchan *et al.*, 1998; Yeh *et al.*, 1998).

Several recent studies have shown that selected alkylphenols, particularly nonylphenol and nonylphenol ethoxylate (NP1EO), possess oestrogen-mimicking capabilities, with effects being found on the reproductive organs of several fish species (Bennie *et al.*, 1997; Blackburn *et al.*, 1999; Bolz *et al.*, 2000; Castillo *et al.*, 1999; Davi & Gnudi, 1999; Ding *et al.*, 1999b; Field & Reed, 1999; Lye *et al.*, 1999; Mann & Boody, 2000; Plomley & Crozier, 1998; Plomley *et al.*, 1999; Shang *et al.*, 1999b; Willetts *et al.*, 1999). The rivers Tees, Aire and Mersey all have concentrations above the threshold level to affect hormonal control of some fish (Blackburn *et al.*, 1999). Chronic oestrogen exposure not only affects the mature reproductive organs, but also has a marked effect on the embryonic, larval and juvenile stages of fish (Lye *et al.*,

1999). In addition is should be noted that NPEs are also used in spermicides, proving a direct pathway for human exposure, primarily accumulating in the liver and kidneys (Hosea & Guengerich, 1998). The oestrogenic potential of nonylphenol polyethoxylates tends to decrease with increasing ethoxylate chain length (Bennie *et al.*, 1997).

Nonylphenol ethoxylates not only have effects on fish reproduction but, together with several other pollutants, have also been linked to an increase in reproductive problems in humans in the western hemisphere (Sharpe *et al.*, 1995, as cited in Willetts *et al.*, 1999; Soto *et al.*, 1991, as cited in Navas *et al.*, 1999). As an oestrogen-mimicking substance, nonylphenol has been shown to be three times more potent than DDT (Soto *et al.*, 1991, as cited in Blackburn *et al.*, 1999).

Due to the toxic effects and their slow biodegradation, the use of NPEs in formulations such as household detergents, has been voluntarily discontinued in North America and some European countries (Bennie *et al.*, 1997; Castillo *et al.*, 1999; Shang *et al.*, 1999b; Zanette *et al.*, 1996) being replaced instead by aliphatic ethoxylate alcohols (Crescenzi *et al.*, 1995b; Lizotte *et al.*, 1999). In these cases NPE use has been restricted to industrial preparations only, as here they are more difficult to substitute due to the requirement of their specific properties (Manzano *et al.*, 1999; Marcomini *et al.*, 1990, 1998). Environmental legislation now also stipulates that surfactants with less than 90 % biodegradability cannot be marketed (Martínez-Barrachina *et al.*, 1999). Signatories to the Oslo and Paris Conventions (OSPAR) (PARCOM recommendation 92/8), which include the UK, have agreed to phase out the use of NPEs in industrial detergents by the year 2000, and hence levels in the environment are likely to decline (Blackburn *et al.*, 1999). Nevertheless, due to the high adsorption potential and low biodegradability of these compounds, it is likely that once entrained in the sediments they are likely to be preserved indefinitely (Shang *et al.*, 1999b). In Canada, nonylphenol and nonylphenol ethoxylates have been added to the secondary Priority Substances List (PSL2) (Bennie *et al.*, 1997), and thus are controlled substances. The legislative decisions remain controversial as a review of the environmental fate and safety

of nonylphenol ethoxylates by Naylor (1995) states that NPEs are 'highly biodegradable, do not accumulate in water, sediment or aquatic organisms and do not pose a credible threat to the environment'.

2.1.3 Radioactivity

When waste LS cocktails are disposed of they invariably contain both organic solvent waste and radioactivity. The major contributor to LS cocktail waste is the biomedical sector, with waste containing mainly ^{3}H, ^{14}C and ^{35}S. The nuclear industry is a relatively minor contributor with many possible radionuclides represented in their waste cocktail, including ^{3}H, ^{14}C, ^{32}P, ^{125}I, ^{131}I, ^{35}S, ^{45}Ca and ^{63}Ni. Activities involved depend on the source, but are generally 1 MBq per 60 L bin, with each bin containing about 10 L of LS cocktail.

Table 2.1 Annual exposure of individuals in the UK from all sources of radiation (McDonnell, 1996)

Source type	*Source specifics*	*Average annual dose (μSv)*
Natural	Cosmic radiation	260.0
	Gamma radiation from he ground and building materials	350.0
	Internal exposure (e.g. ^{40}K in food)	300.0
	Radon	1300.0
Artificial	Discharges from nuclear sites	0.4
	Fallout from past weapons testing	5.0
	Medical exposures	370.0
	Occupational exposures	7.0
	Radioactivity in products (e.g. smoke detectors etc.)	0.4
Total (rounded)		**2600.0**

Radioactive materials can be broadly classified as man-made or natural. Naturally occurring radionuclides are either present due to their long half-lives (primordial radioisotopes) or due to their continual production by natural processes. Examples of primordial radionuclides include ^{238}U (4.5 billion year half-life) and ^{232}Th (14 billion year half-life). Included in the readily replenished radionuclides category are ^{3}H and ^{14}C (Roberts, 1998). Exposure to low levels of irradiation from natural environmental and cosmic sources has occurred

throughout geological time (Templeton, 1976). Since 1945 and the start of large-scale nuclear testing, extensive defence and industrial use of radionuclides has resulted in ever increasing levels in the environment (Thiessen *et al.*, 1999; Whicker *et al.*, 1999). To compare the relative exposure as a result of specific exposure types see Table 2.1. The National Radiological Protection Board recommends a maximum annual exposure of 0.3 mSv per member of the public (McDonnell, 1996).

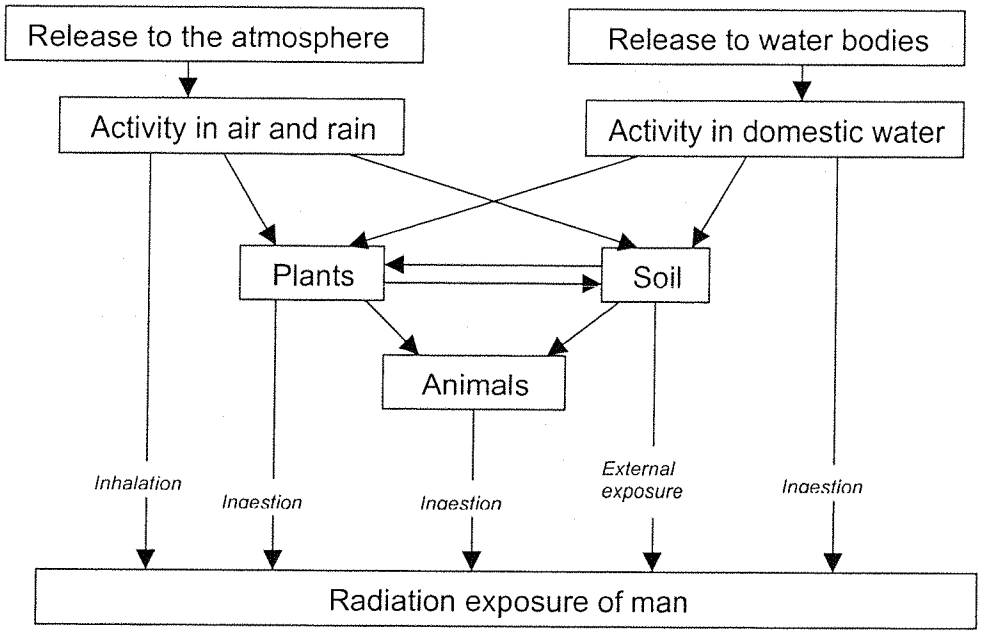


Figure 2.7 Outline of major transfer routes in the migration of radionuclides in the terrestrial environment (Source: Thiessen *et al.*, 1999)

As a result of the many ways in which man can be exposed to radiation (Figure 2.7), the management and safe disposal of all forms of radioactive waste is now a world-wide issue (Roberts, 1998). Although definitions of the classification vary from country to country, radioactive waste is generally classed as high level waste (HLW), medium level waste (MLW) or low level waste (LLW), where the sum of the radionuclide fractions and its origin is used to determine the class (Tsoulfanidis & Cochran, 1991). Liquid scintillant cocktail waste falls into the LLW category, where the radioactivity decays away to trace levels within 300 years (Ginniff, 1987). About 10 % of LLW is from hospitals, medical schools, research laboratories and universities, and a large proportion of this waste is made up of liquid scintillant cocktails (Roberts, 1998). Industrial and

institutional LLW includes ^{3}H, ^{14}C, ^{60}Co, ^{137}Cs, ^{99}Tc, and ^{22}Na (Tsoulfanidis & Cochran, 1991). As in the case of liquid scintillant waste, when radioactive waste is mixed with other toxic or hazardous waste, it has to be classified as 'mixed' waste, and as such requires specific disposal methods (Roberts, 1998).

A million cubic metres of low-level waste (LLW) has thus far been disposed of in the UK (Nirex, 1998). Whilst removal mechanisms for tritium in water have been developed, they tend to be very expensive, and therefore if the concentration is sufficiently low, the waste can be considered for dispersal in a large body of water (Roberts, 1998). Any LLW disposal requires some sort of authorisation. If any of the liquid fraction of this waste is to be disposed of into the sewers, authorisation must be sought from the Environment Agency. Generally this disposal route is only used for liquid waste effluent from clinics, hospitals and universities (Roberts, 1998). In the United States, the Nuclear Regulatory Commission specifically dictates that water treatment plants are used for the disposal of ^{14}C and ^{3}H wastes (Klein & Gershey, 1990). As radionuclides are discharged into the aquatic environment, the exposure of individuals to radionuclides both transferred up the food chain and ingested directly, are both of interest and importance. The law dictates that a thorough assessment of the radiation dose to the public resulting from radioactive waste discharge has to be performed. In order to assess the safety of discharges, additional information on the characteristics, uses and possible consequences of the radioactive source have to be established.

For an accurate assessment of doses to the public, modelling is necessary to determine the pathways of radionuclides following disposal. This assessment needs to include considerations of water and atmospheric dilution factors, half-lives and percentage activity transfers at each stage. Virtually every radionuclide with a half-life of a few days or more has been studied with regards to its environmental behaviour (Whicker *et al.*, 1999).

2.1.3.1 Environmental Radiation Effects

Despite constant exposure to background radiation, organisms can still be sensitive to even very low levels of irradiation. The International Commission on

Radiological Protection (ICRP) has adopted a linear response relationship between dose and effect (Cox *et al.*, 1995; Hamilton, 1985), however this is greatly disputed. It does however mean that there is no such thing as a threshold dose below which no effects occur (Hamilton, 1985). High mortality of fish eggs in the presence of radioactivity has been found, and it is predicted that even the introduction of extremely low levels of radionuclides into the marine environment could have deleterious effects on yields from commercial fisheries (Polikarpov, 1966, as cited in Templeton, 1976). This impact will be particularly severe in very simple foodwebs where energy pathways cannot easily be adapted. In addition to the somatic affects on fish eggs, it is generally accepted that long-term radiation exposure can also result in the artificial induction of mutations, thus increasing the frequency of the formation of chromosomes with detrimental effects (Templeton, 1976).

It should be noted that as for the contaminants discussed in previous sections, radioactivity, assuming a sufficient half-life, also has the capacity to bioaccumulate and be passed through the food web. Hutchins *et al.* (1996a) showed that environmental factors have an impact on the radionuclide retention in organisms. The researchers found that ^{241}Am had a biological half-life in the sea star *Asterias forbesi* of 31 days at 12 °C, whereas it was virtually infinite at 2 °C. This suggests that ingested radionuclides would be far more effectively retained in higher latitudes than in the tropics. Another study by the same authors (Hutchins *et al.*, 1996b) showed that at lower temperatures the brittle star *Ophiothrix fragilis* showed significantly reduced uptake of radionuclides but no reduction in biological half-life.

Generally exposure to ionising radiation can be linked to an increase in occurrence of cancer and the appearance of hereditary diseases in the offspring of those exposed (Cox *et al.*, 1995). Most of this information has been collected from life span studies on occupationally exposed workers and the Nagasaki and Hiroshima atomic bomb victims of 1945. There is generally a minimum time between irradiation and the onset of any radiation-induced tumours. This time is termed the latent time, it is a function of age and tumour type and can last from a few years to tens of years. The Oxford Survey of Childhood Cancer found a

40 % increase in childhood cancer in children up to 15 years of age following *in utero* doses of 10 – 20 mGy. Several studies have also shown an increased incidence of leukaemia in occupationally exposed workers. The lowest acute dose required to give a significant increase in tumour occurrence in mice and adult humans was shown to be as low as 100 – 200 mGy (Cox *et al.*, 1995).

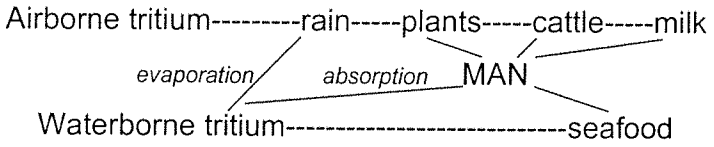
2.1.3.2 Tritium

A major radionuclide present in LS cocktail waste is tritium (^{3}H). Tritium can be produced by the action of cosmic rays on the earth's atmosphere, and hence occurs naturally. The global inventory of naturally occurring tritium is estimated to be 1.3×10^{18} Bq (3.6 kg), and it is formed at an annual rate of about 70×10^{15} Bq (0.2 kg) (Higgins *et al.*, 1996). Despite significant background levels, tritium concentration in the environment has been greatly increased by nuclear testing (Passo & Cook, 1994), the nuclear industry (Fairlie, 1992) (releases shown in Table 2.2), and industrial, commercial and research uses (Hill & Johnson, 1993). Present contributions from all industrial activities contribute approximately 25 % to the naturally produced atmospheric tritium (Higgins *et al.*, 1996).

Table 2.2 Annual tritium emissions from UK nuclear plants (from Fairlie, 1992)

Nuclear facility	*Tritiated water vapour admissions to atmosphere (TBq/yr)*	*Tritiated water discharges to sea (TBq/yr)*
AERE Harwell	46.0	1.8
Amersham International (Amersham Plant)	14.0	6.0
Amersham International (Cardiff Plant)	180.0	601.0
AWRE Aldermaston	100.0	0.1
Chapelcross	1900.0	0.3
Dungeness B AGR	3.2	7.3
Hartlepool AGR	3.2	166.0
Heysham 1 AGR	3.2	95.0
Hunterston B AGR	8.2	333.0
Sellafield	593.0	1699.0
UKAEA Dounraey	18.0	9.9
UKAEA Winfrith	8.4	149.0
Wylfa Magnox	13.0	3.0

Most of the tritium in the environment is in the form of tritiated water, where ^{3}H replaces ^{1}H. Following atmospheric release of tritium in its gaseous form it is rapidly converted to tritiated water (HTO) by oxidation and isotopic exchange (Higgins *et al.*, 1996). In this new form it can enter the body by ingestion, inhalation or skin absorption (Hill & Johnson, 1993). The schematic below is a very simple representation of the major exposure pathways of man to tritium.



Tritium has a half-life of 12.26 years, decaying to ^{3}He, with the emission of a beta particle (Hill & Johnson, 1993; Passo & Cook, 1994). The beta emissions have a maximum energy of 18.6 keV and cannot penetrate intact human skin due to their small range in tissue (~ 6 μm), and as such they pose no external hazard (Hill & Johnson, 1993). Tritium is readily incorporated into biomolecules such as DNA, with intermolecular exchange of tritium resulting in its assimilation (Hill & Johnson, 1993). Studies have indicated that selective assimilation of tritium may occur into cell nuclei, resulting in elevated concentrations, particularly in nuclear proteins (Fairlie, 1992). When the tritium is organically bound, it is approximately twice as radiotoxic (Higgins *et al.*, 1996). It will not only deliver radiation doses over a much longer period of time than when the tritium is in a purely aqueous form (due to the longer biodegradation half-life), but will also act on a more organ specific basis. Organically bound tritium is of particular concern where there is rapid cell division, such as in the reproductive organs and bone marrow (Fairlie, 1992).

With the relative abundance of tritium in the environment, a large number of studies have been undertaken to investigate the effects of this radionuclide, and provide more information on the mechanisms by which it travels through different media. Several inferences have been made as to the direct relationship between tritium levels in the environment and birth defects and prevalence of certain cancers.

The Atomic Energy Board of Canada carried out a study analysing birth defects around the Pickering nuclear power station near Toronto, Ontario (Fairlie, 1992). The reactors emit similar amounts of tritium to the Sellafield reprocessing plant (~2500 TBq/year), with tritium being the only radionuclide emitted into the atmosphere in appreciable quantities. The study found an 80 % increased occurrence of children born with Down's Syndrome at the nearby town of Pickering, and a 46 % increase at Ajax, a town further from the nuclear power station. This zoning of birth defects showed a direct correlation with airborne tritium releases in the case of Pickering, and ground monitored tritium levels in Ajax (Fairlie, 1992). Further studies also suggest a relationship between high tritium levels and an increased mortality from specific cancers, e.g prostatic cancer.

2.1.3.3 ^{14}C

Another radionuclide used extensively in industry and one of the major radioactive constituents in most liquid scintillant cocktail waste is ^{14}C. In global environment terms, ^{14}C is derived from three main sources: natural production, atmospheric testing of nuclear weapons and the nuclear fuel cycle. In addition, Nycomed Amersham is also a major contributor to levels in the coastal marine environment as a result of its production of labelled compounds for use in research and medicine (Cook *et al.*, 1998). Natural ^{14}C levels fluctuate slightly over a hundred- and thousand-year cycle. By 1963, anthropogenic inputs to the environment as a result of extensive nuclear testing and power production resulted in an atmospheric concentration of ^{14}C that was twice the natural levels. Since this time, with partial nuclear testing bans in place, atmospheric levels are decreasing as ^{14}C is drawn down from the atmosphere into the oceans (Cook *et al.*, 1998).

Uptake during photosynthesis incorporates the radioactive carbon into plant matter. Subsequent consumption of the plants results in the radionuclide being passed up the foodchain. Human exposure is not solely through ingestion of ^{14}C contaminated food, but also through external radiation, and internal radiation following inhalation (Cook *et al.*, 1998). The radiological importance of ^{14}C is

primarily as a result of its environmental mobility and relatively long half-life (5730 years), thus facilitating bioaccumulation.

2.1.4 Conclusion

It is with these factors in mind that concerns have been raised over the sewer disposal of many industrial waste products, including LS cocktails. The accumulation and disposal of radioactive waste in the UK is controlled by the enforcement of the Radioactive Substances Act (1993). Several legislative changes have also been introduced to regulate water pollution, such as the Water Resources Act (1991) in the United Kingdom and the Federal Water Pollution Control Act (1972) in the United States. As a result of this legal framework, more stringent regulations on effluent discharge into controlled waters are now in place.

Waste solvents and solvent-containing wastes cannot be disposed of to either surface water or sewers without a disposal licence. Authorisation can only be attained from the relevant water authority or the sewage authority in Scotland (Department of the Environment, 1977). Initial authorisation for the sewer disposal of Gold Star™ and EcoSafe™ was issued by Thames Water to National Diagnostics of the USA, after tests were carried out on di-isopropyl naphthalene, indicating it to be miscible with water and biodegradable. Subsequently the water authority was notified of the presence of other non-soluble elements of the LS cocktails, namely PPO and bis-MSB, authorisation for sewer disposal was withdrawn (Smythe, *pers. comm.*, 2001), and an alternative route had to be sought.

2.2 Waste disposal by incineration

An alternative to sewer disposal is the use of incineration for LS cocktail waste, as is carried out at Rechem International Ltd., Southampton, Grundon (Waste) plc., Slough, and the Queen Alexandra Hospital, Cosham near Portsmouth. Incineration is used for large volumes of solvent based and radioactive clinical and research waste (Collins & Daugulis, 1999) as it eliminates biological and chemical hazards, along with reducing the volume of waste for burial in landfill sites by up to 93 % (S. Grundon (Services) Ltd., 1998a). Despite a reduction in

waste, incineration also produces large volumes of gaseous emissions such as polyaromatic hydrocarbons, dioxins, and acid gases (SO_2 and HCl) along with particulates in the form of heavy metals and dust (Friends of the Earth, 2000; Rubin, 1999; Schuhmacher *et al.*, 1998). The emission of these toxic organic compounds is greatly influenced by the waste type and operating conditions of the incineration facility (Chagger *et al.*, 2000; Dugenest *et al.*, 1999), with reduced emissions at higher operating temperatures.

Incinerators generally consist of a primary and secondary combustion chamber. Incineration of waste involves the heating of the product by radiation from the furnace and exchange with hot combustion gases, resulting in volatilisation of the waste and evolution of gases. The intention of the secondary combustion chamber is to fully pyrolyse these waste gases before they are released into the atmosphere. Often the gaseous products are formed in a region of the incinerator where insufficient oxygen and heat are available for complete combustion to occur. This can then result in the formation of hazardous or toxic compounds such as dioxins and polyaromatic hydrocarbons. The widest range of toxic gases is produced during the start-up phase of the plant (Chagger *et al.*, 2000).

The recently introduced Integrated Pollution Protection Control (IPPC) guidelines regulate atmospheric and terrestrial emissions. They stipulate the minimisation of toxic flue gases, leached material and residual material from flue gas cleaning. Particular attention is focussed on the heavy metal residues and production of dioxins (Tsiliyannis, 1999). European controls on waste incineration emissions will also be further tightened by the imminent introduction of the EC Directive on Waste Incineration (ENDS, 1999). This directive will enforce minimum incineration temperatures of 850 ^{o}C and a 3 % maximum total organic content of the ash and slag (European Union, 2000).

2.2.1 Ash and particulates in flue gas

Inorganic products of the combustion process may accumulate in the ash or be emitted as particulates in the flue gas (Yang *et al.*, 1998), and as such may be subjected to hazardous waste notification procedures (Department of the

Environment, 1977). The solid incineration waste is generally trapped in the bottom of the kiln as bottom ash or in the towers as fly ash. This ash not only offers a matrix and chemical composition to trap combustion products, but also acts to immobilise any radioactivity present in the incinerated waste (Tsoulfanidis & Cochran, 1991).

The accumulation of inorganic and non-pyrolysed organic fractions occurs particularly when barrels of waste are initially introduced into the incinerator. Following the introduction of a barrel a temperature decrease occurs leading to a reduction in the organic fraction burnt, a release of products of incomplete combustion (Dugenest *et al.*, 1999; Wei & Lee, 1998) and large volumes of organic carbon dust (Chagger *et al.*, 2000). The particulates are generally toxic, persistent and bio-accumulative substances. They can act to exacerbate dermatitis (Nethercott, 1981, as cited in Bener *et al.*, 1998), and lung diseases like asthma and bronchitis. The presence of heavy metals such as cadmium and mercury can have additional deleterious effects on the kidneys and nervous system (Friends of the Earth, 2000). Added complications arise when the incinerated waste contains halogens, sulphur, nitrogen, phosphorus (Department of the Environment, 1977), or volatile radionuclides. Further details of specific solid waste produced by the incineration of liquid scintillant waste are given in Section 2.3.1 below.

2.2.2 Polyaromatic hydrocarbons (PAH) and dioxins

Polyaromatic hydrocarbons are formed by pyrolysis and/or carbonisation whenever organic materials, such as solvents, are burnt (Heitkamp & Cerniglia, 1989; Rockne & Strand, 1998; Wei & Lee, 1998; Wilson & Jones, 1993). It is predominantly the more volatile PAH that are detected in the flue gas, with largest quantities being emitted at the low combustion temperatures of start-up and barrel introduction (Chagger *et al.*, 2000). As mentioned in Section 2.1.1.2, PAH constitute a group of priority pollutants that have been found to be carcinogens and mutagens. Along with their toxicity, they are also relatively stable compounds which are partially resistant to biodegradation (Heitkamp & Cerniglia, 1989; Jee *et al.*, 1998; Law *et al.*, 1997; Martens & Frankenberger, 1995; Wilson & Jones, 1993).

Together with the production of PAH by combustion, dioxins are also often emitted from the incinerator in the form of cooling stack gas or fly ash (Chagger *et al.*, 2000; Fairey *et al.*, 1997). Dioxins are produced when chlorine-containing waste is burnt at low temperatures (Vikelsøe & Johansen, 2000), with fly ash particles catalysing the formation reactions (Addink & Olie, 1995, as cited in Chang & Huang, 1999). Dioxins are hydrophobic, very toxic, persistent and have a tendency to bioaccumulate in various media (Jones & de Voogt, 1999; Schuhmacher *et al.*, 1998, 1999; Yoshida *et al.*, 2000). According to a draft report produced for the US EPA, they can be associated with cancer, endometriosis, and reduced sperm counts, immune system and foetal development (Friends of the Earth, 2000; Yoshida *et al.*, 2000). In Japan approximately half of all dioxin intake by the general public is due to fish ingestion (Yoshida, *et al.*, 2000). Human dioxin levels in the UK and most other areas of Europe will not be as high due to fish constituting only a relatively small proportion of the diet.

The emission of dioxins and PAH causes controversy in the siting and building of new incinerators (Schuhmacher *et al.*, 1999; Yoshida *et al.*, 2000). Both PAH and dioxins are often enriched in the fly- and bottom ash produced by the incineration process (Wei & Lee, 1998). Chang & Huang (1999) showed that the air pollution control devices, designed to minimise emissions of toxic flue gases such as dioxins, may actually have the opposite effect, with their content in fly ash increasing on passing through these devices. PAH and dioxins released into the atmosphere from incineration stacks are usually done so at a specific temperature to ensure that they rise and are extensively diluted by dispersion in the atmosphere. Subsequent aerial deposition can result in their incorporation in soil, plants and water (Lorber *et al.*, 1998; Schuhmacher *et al.*, 1999). A study by Schuhmacher and co-workers (1998) showed only low levels of contamination in soil samples taken from around the incinerator. This was also found by Lorber *et al.*, (1998), who additionally found elevated concentrations at 2 km downwind of the site, suggesting that exit velocity of the stack emissions carried gases beyond the perimeter fence before deposition occurred. When buildings are situated in the emission plume pathway, pollutants have been

shown to be caught in the turbulence around these buildings affecting their dispersion and causing downwash to ground level, therefore having a significant impact on local air quality (Kayin *et al.*, 1999). PAH are carried long distances by air in the troposphere whilst attached to particulate carbon (Henner *et al.*, 1997), and can therefore have an influence even at great distances from their source.

The Clean Air Act Amendments of 1990 require the control of hazardous air pollutants (Cano *et al.*, 1999) such as dioxins and PAH. If combustion of these toxins is to continue, then newer, more efficient incinerators, releasing fewer PAH, need to be developed. New incinerators, such as the consolidated incineration facility, are currently being tested for the disposal of hazardous, radioactive and mixed waste. Auxiliary fuel is fed into both chambers to ensure the maintenance of a minimum temperature, so that the primary chamber has an operating temperature of 1600 °F and the secondary chamber is held at approximately 1800 °F (Birk, 1998).

2.2.3 Carbon dioxide

Along with PAH and dioxins, the releases of carbon dioxide as an incineration waste product has lowered the worldwide public acceptance of incineration as a waste disposal mechanism (Chang & Huang, 1999; Schuhmacher *et al.*, 1999; Singleton, 1994; Sonesson *et al.*, 2000; Vanderberg, *et al.*, 1999). Despite this, the number of incinerators has increased in recent years due to a lack of available landfill sites (Schuhmacher *et al.*, 1998).

Incineration of 1 L of LS cocktail waste produces 1804 L of carbon dioxide. Hence on the basis of 80,000 L of waste, incineration has a total annual contribution of 144.3 million litres of carbon dioxide. Although this is a relatively minor source, the development of an alternative disposal method, which produces less atmospheric carbon dioxide, would be welcomed.

2.2.4 Costs

In addition to the environmental impacts that have been discussed, incineration is not necessarily a cost effective disposal mechanism. The cheapest disposal

mechanism is that of sewer disposal assuming no effluent clean up is required, and licence fees are minimal. The cost of incineration is between £ 35 and £ 200 per 60 L bin depending on the incineration facility used (Sealy, *pers. comm.*, 2001; Smythe, *pers comm.,* 2001*)*. Each bin can hold 1000 large plastic liquid scintillation vials, containing a total of about 10 L of LS cocktail (Smythe, *pers. comm.*, 2001).

Further to the incineration costs, following the Lord Marshall report in 1998 a Climate Change Levy will be introduced in April 2001 to tax business use of energy. The aim of this tax is to reduce UK greenhouse gas emissions (HM Treasury, 2000). In addition it is anticipated that some of the revenue from the levy can be used to credit business investment, through reduced National Insurance contributions (0.3 %), acting to boost the economy in the long term. The remainder of the revenue (£150 million) will be used to promote energy efficiency and support renewable energy (Anon, 1999c; HM Treasury, 2000). The government has offered tax rebates of up to 50 % to the ten most energy-intensive sectors on the condition that they sign up to energy efficiency schemes (Anon, 1999b). The tax will reflect the carbon content of the different fuels per unit energy. The charges, as follows, were set out in the 2000 Finance Bill:

Coal	0.15 p/kWh
Natural gas	0.15 p/kWh
Liquid petroleum gas	0.07 p/kWh
Electricity	0.43 p/kWh

(HM Treasury, 2000)

This rate will not apply to fuel used by the domestic or transport sectors. In addition it will not be levied on oil, as this is already subject to mineral oil duty. All in all this new levy will result in the incineration option being even more expensive as costs are more than likely to be passed on to users.

2.3 Case study: liquid scintillant cocktail incineration in Slough

S. Grundon (Waste) plc. is the largest privately owned waste management group in Britain with a remit that includes:

- high temperature clinical waste incineration
- hazardous waste transfer and treatment, and
- environmental monitoring of the licensed waste facilities.

For the purpose of this case study the discussion will be based around their clinical waste incineration facility in Colnbrook, Slough, Berkshire, which was opened in 1991, and upgraded to comply with the EC Hazardous Waste Incineration Directive (94/67/EC) in 1997. The waste processed at the Colnbrook site is predominantly solid and is stored in plastic bins or sacks. It is transported to Grundon plc. in refrigerated vans and trucks where it is processed as soon as possible, storage being for a maximum of 8 weeks (Wakerley & Davison, 1994).

2.3.1 The incineration process and waste products

The maximum capacity of the incinerator is 720 tonnes/month (Wakerley, 1996). Limits on quantities that can be processed are dictated by the volume of low-level waste ash produced (Wakerley, 1996). Waste processed at the site comes primarily from research institutions and hospitals in and around London, with about 5 % of the total volume being radioactive. The hospital radwaste consists mainly of ^{14}C, ^{51}Cr, ^{57}Co, ^{58}Co, ^{99}Tc, ^{125}I and ^{131}I, and the radioactive waste from the research institutes is primarily ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{45}Ca and ^{125}I (Wakerley & Davison, 1994). Waste liquid scintillant cocktails contained in plastic or glass liquid scintillant vials are not distinguished from the bulk waste and constitute but a fraction of the total (Waverley & Davison, 1994).

The incinerator system consists of several conveyor systems, a primary and secondary combustion chamber, heat exchanger, effluent treatment system, extractor fan and a 38 m stack. Radioactive waste is interspersed with non-radioactive waste prior to incineration to reduce the radioactive concentration of the ash produced. The waste is introduced into the primary combustion chamber on a conveyor belt, where there is a minimum residence time of 8 hours and pyrolysis takes place. The conditions in this chamber may be air deficient (Frith, 1993). It is here that the waste is broken up to ensure that all surfaces are exposed. Pyrolysis gases from this chamber pass into the

secondary chamber for further combustion, where they remain for a minimum of 2 seconds. The temperature in this chamber is at least 1000 °C before any waste is introduced (S. Grundon (Services) Ltd., 1998b). Exhaust gases then pass through the heat exchanger where energy is removed by a jet of aerosols, reducing the temperature to around 250 °C. In the scrubbing column the gases are further quenched using an alkaline scrubbing solution producing final exhaust gases which pass on to the stack and particulates which are retained in the settling tank. The final exhaust gases are reheated to 110 °C to aid steam plume formation and are released to the atmosphere (S. Grundon (Services) Ltd., 1998b). Table 2.3 gives details of waste products from the incineration process.

Table 2.3 Waste products and disposal route from the incineration process

Waste type	*Specifics*	*Contaminants contained*	*Volume produced (m^3) / unit / month*	*Disposal route*	*Reference*
Gaseous	Exhaust gases	^{3}H, ^{14}C, particulates, SO_2, HCl, HF, NO_x, dioxins, Cd, Hg, Zn	1.2×10^7	Atmosphere	Wakerley, 1996 & Frith, 1993
Liquid	Cleaning / scrubber liquid	^{125}I, ^{131}I	78	Sewage treatment plant (dilution factor 40,000 in Thames River)	Wakerley & Davison, 1994
Solid	Ash	^{32}P, ^{35}S, ^{45}Ca, ^{51}Cr, ^{57}Co, ^{58}Co, ^{99}Tc	114	Closed skip to landfill	Wakerley, 1996
	Metal residues / glass beads		0.001	Closed skip to landfill	Wakerley & Davison, 1994
	Spent resin	Hg, Cd	0.04	Closed skip to landfill	Wakerley & Davison, 1994
	Spent filters			Incinerator	Wakerley & Davison, 1994

It should be noted that temperatures used in this incineration facility are not as high as those recommended for minimum operating temperatures in the primary and secondary combustion chambers (1600 and 1800 °C respectively). These

elevated temperatures are used to ensure optimum reduction in toxic product gas emissions such as dioxins and PAH (Birk, 1998).

The waste ash, up to 30 m^3 (Frith, 1993), can only be held on-site for a maximum of 28 days, after which it has to be transported to a controlled landfill site. It can only be disposed of by landfill if it does not contain any alpha emitters, the total activity is less than 400 000 kBq/m^3, and the single item activity is less than 40 000 kBq/m^3 (Hudson, 1997). As for the gaseous releases, carbon monoxide levels in these effluents may not exceed 50 mg/m^3 (Frith, 1993).

2.3.2 The incineration of liquid scintillant cocktails

In July 1998 decisions were made by Grundon (Waste) plc. to accept some low level radioactive waste from the Trident nuclear missile programme at the HM Clyde Naval Base in Scotland. It consisted of waste, including liquid scintillant cocktails, from laboratory tests carried out on radioactive contaminated packaging material. The 6 x 60 L drums per year of liquid scintillant cocktails involved were obtained from Canberra Packard Ltd. who have a contract with Grundon (Waste) plc. for the incineration of their low level waste.

Following several letters between the Scottish Environmental Protection Agency (SEPA), the Ministry of Defence (MoD) and the Health and Safety Executive (HSE), a request was made by SEPA in early 1999 to Slough Borough Council for comment on the MoD proposal. Slough is a nuclear-free town with a high population density. A public consultation was started and a consultation document was drawn up. This document pointed out that Grundon (Waste) plc. already has authorisation for the disposal of radioactive clinical waste with an environmental impact assessment (EIA) already having been carried out. In addition, the MoD waste would only be a small fraction of the total radioactive waste that is incinerated at the Colnbrook site, containing only very low levels of tritium (Ellis, 1999). This would be the first military radioactive waste to be accepted by Grundon (Waste) plc.

There was a public outcry in response to the proposal, with several newspaper articles appearing in the local and national press (see Appendix 2). On the 4th of March 1999, Slough's Development Committee voted to reject the proposal and passed this recommendation back to SEPA to make the final decision. As a result of intense public pressure and media coverage (Dutt, 1999), in August 1999 Grundon (Waste) plc. informed the MoD that it would not accept the liquid scintillant cocktail waste for incineration in Slough. The decision was also based on the fact that the MoD could not class the waste as clinical waste, only stating it as being radioactive. Grundon (Waste) plc. hold a licence for only the incineration of clinical waste.

2.3.3 Conclusion

The case of the Slough incinerator-MoD waste proposal indicates that the public are very wary of nuclear waste, and are also greatly opposed to incineration as a disposal mechanism. In addition to the financial factor, it is quite clear that when these two issues are combined, such as in the case of waste liquid scintillant cocktail disposal, an alternative to incineration must be found.

2.4 The use of biodegradation

Biodegradation is the natural process whereby microorganisms alter and break down organic molecules into less complex substances. It is one of the environment's self-purification mechanisms (Bartha & Atlas, 1987; Brown *et al.*, 1999). Several terms are regularly used in conjunction with biodegradation. Primary biodegradation is the transformation of a substance by microorganisms resulting in the loss of its chemical identity. Mineralisation, which is also known as ultimate biodegradation, is the breakdown of a chemical substance to carbon dioxide, water and mineral salts. This is generally accompanied by the production of new biomass and organic microbial biosynthesis products. The stages between primary and ultimate biodegradation are termed partial biodegradation. The tranformation of a chemical substance by microorganisms resulting in the loss of a specific property is known as functional biodegradation. If an environmental concentration threshold has been established for the substance in question and if at any stage of the biodegradation process this

threshold is attained, then environmentally acceptable biodegradation is said to have been attained (OECD, 2001).

Many organic compounds are mineralised for the production of energy and carbon for growth by this means. When all relevant enzymes are available, and under optimised conditions, final products include fatty acids, water, aliphatic compounds, carbon dioxide and bacterial biomass (Atlas, 1995; Bartha & Atlas, 1987; Mohammed *et al.*, 1996; Quiroga *et al.*, 1999). The bioavailability is of primary importance in determining a pollutant's biodegradation potential. It is therefore this factor that limits the biodegradation of pollutants such as petroleum hydrocarbons with low water solubility and dissolution rates, resulting in correspondingly low bioavailabilities (Page *et al.*, 1999). In addition a supply of electron acceptors, such as oxygen, nitrate or sulphate, is required to ensure microbial population growth during consumption of the organic contaminant (Keijzer *et al.*, 1999; Liu & Suflita, 1993).

Bioremediation is the stimulation of biodegradation through the optimisation of conditions for breakdown (Henner *et al.*, 1997; Hoff, 1993; Wilson & Jones, 1993). This stimulation effect can be divided into two categories, namely: biostimulation and bioaugmentation. Biostimulation is where physical and chemical conditions like oxygen and nutrient levels, are optimised for degradation. Bioaugmentation concerns the artificial increase in microbial concentrations to enhance degradation rates, i.e. the introduction of a pre-adapted culture (Buitrón *et al.*, 1998; Margesin & Schinner, 1998; Mohammed *et al.*, 1996). These adapted organisms will have been selectively cultured or genetically engineered for this purpose. Bioremediation can take place either *in-situ* or *ex-situ*. The decision is based on the volumes to be processed, pollutant type and the technique chosen. Generally the technology is seen as cheap, simple, environmentally sound, publicly acceptable and safe. However, limitations include the time taken and a lack of data regarding long-term effects and target pollutants (Mohammed *et al.*, 1996).

The harnessing of organisms to transform a wide range of organic substrates has been used for over a hundred years in the treatment of wastewaters. The

key to reducing environmental impact resulting from down-the-drain chemicals has long been their processing in wastewater treatment plants (Schröder *et al*, 1999). Over the last few decades biodegradation has also been increasingly employed for the remediation of contaminated soil, and the clean up of pollutant spills (Brubaker & Stroo, 1992; Mohammed *et al.*, 1996; Shuttleworth & Cerniglia, 1995; Tang *et al.*, 1998). By 1999 the US EPA had already spent about 15 billion dollars on contaminated land remediation, most of which was used in the clean up of organic chemical contaminated hazardous waste sites (Singleton, 1994).

Complete degradation products may be harmless whereas incomplete degradation can also occur, resulting in metabolite intermediates that are sometimes more toxic and hydrophilic than the parent compounds. It is also worth noting that not all organic chemicals can be broken down by degradation. Problems are most often found with the wide variety of foreign synthetic compounds which are not easily recognised by existing degradative enzyme systems (Singleton, 1994).

Biodegradation is not only used for clean up methods but also as a standard test for environmental impacts of chemicals being considered for disposal (Žgajnar Gotvajn & Zagorc-Končan, 1999). One of the methods used by the US EPA for this purpose is Method 304B, which uses activated sludge to biodegrade the chemical-containing effluent. The test is set up in a flask with a sealed headspace to trap any gases emitted by the metabolism. These gases are then tested and measured in order to quantify biodegradation of the effluent. Method 304A is a similar system, but here there is continuous venting, direct to an analyser (Cano *et al.*, 1999).

On an international scale, the most widely used test to estimate removal and biodegradation in activated sludge systems is the OECD Confirmatory test (Shimp & Larson, 1996). As for EPA methods, this test employs carbon dioxide evolution trapped in a sealed vessel to quantify mineralisation. This test has been adapted several times for specific purposes, e.g. the modified Sturm Test (OECD 301B) and OECD 301F (van der Zee *et al.*, 1998).

The tests conducted for 'readily biodegradable' include the protocols defined in OECD 301A-F. To be classified as readily biodegradable by these OECD tests, a compound must show at least a 60 % theoretical carbon dioxide loss within 28 days, 10 % of which must occur in the initial 10 days (Staples *et al.*, 1999; Žgajnar Gotvajn & Zagorc-Končan, 1999). Although this classifies substances as being readily biodegradable, the stringent nature of these tests does not automatically exclude other substances from being biodegradable in practice. The OECD definition for substances that exhibit sufficient degradation to suggest that they will not be persistent in the environment is that of 'inherently biodegradable'. Tests can be conducted on the standard 28 days timescale, in which case either a daily degradation of greater than 20 % or a total degradation of greater than 70 % in 28 days is used. Alternatively, prolonged exposure or more favourable test compound biomass ratio is used to create more favourable test conditions. Inherent biodegradation tests are described in the protocols given in OECD 302A-C. If chemical substances do not reach these thresholds then they can be considered to be non-biodegradable. A final test that may be used is the simulation test, used for confirmation purposes. This is where environmentally relevant conditions are used to mimic the receiving environment of the substances once they are disposed of into the environment (Robinson, 1999). The above-mentioned tests all involve the measurement of substrate consumption, through % dissolved organic carbon (DOC) removal, % carbon dioxide production or % biological oxygen demand (BOD) removal.

Another method to investigate biodegradation is by microorganism growth. This too is used in certain standard tests, but cannot be used to determine biodegradation rate constants (Quiroga *et al.*, 1999). Both the substrate consumption and microbial growth approaches are termed 'indirect methods' as they do not directly quantify the loss of pollutants.

The use of bioremediation to overcome some of the problems with the present disposal techniques for liquid scintillant cocktails is reported in this thesis. The aim of the work was to produce end products following biodegradation that can

be disposed of with less impact on the medium into which they are deposited. In addition there will be a greatly reduced carbon dioxide production, and the resultant carbon will be primarily as biomass which can then be disposed of directly into a large water body.

In addition to a reduction in toxic waste products, once mechanisms of the degradation are thoroughly understood, the technique should be a relatively simple and inexpensive disposal mechanism whereby large volumes of LS cocktail can be processed.

2.5 Previous biodegradation studies undertaken in this field

Bioremediation has been used extensively for several of the compounds present in liquid scintillant cocktails. In addition, related compounds have also been studied. The aim of this section is to outline some of these studies and to indicate the position of this work within the field.

2.5.1 Linear alkyl benzenes (LAB)

Linear alkyl benzenes have been shown to be readily biodegradable. Carbon dioxide evolution was used to determine biodegradation in an OECD 301B test, which showed 67 % biodegradation by day 28 (Hüls, 1987, as cited in Binetti *et al.*, 2000). Studies were also carried out in more natural systems by the Standard River Die-away Test at a LAB concentration of 100 – 500 ppb, where gas chromatography analysis was used to quantify analyte concentration changes. Gledhill and co-workers (1991, as cited in Binetti *et al.*, 2000) showed that a concentration decrease of more than 90 % occurred, with an analyte half-life of 4 – 15 days.

2.5.2 Phenol degradation

Buitrón and co-workers (1998) used pre-adapted bacterial cultures to assess the aerobic biodegradation potential of phenols such as phenol, 4-phenol, 2,4-chlorophenol and 2,4,6-trichlorophenol. Residual phenol concentrations were determined by HPLC. The group concluded that degradation rates were significantly higher when mixed activated sludge cultures were used in preference to single bacterial strains, with 80 % phenol degradation being

achieved in 19 hours rather than 12 days. Phenol degradation has been the object of study for bioremediation research in 8.8 % of all work on organic remediation (Mohammed *et al.*, 1996).

2.5.3 Polyaromatic hydrocarbons

A large number of studies have investigated the biodegradation of polyaromatic hydrocarbons. Nearly 20 % of all studies carried out on organic chemicals have focussed on PAH remediation (Mohammed *et al.*, 1996). It is generally accepted that microorganisms can metabolise both higher and lower molecular weight PAH found in the natural environment (Pothuluri & Cerniglia, 1994). Biodegradation of PAH with less than five rings usually occurs readily under aerobic conditions, however rates are one to two orders of magnitude lower without the presence of sufficient oxygen (Rockne & Strand, 1998). Molecular oxygen is however necessary for the metabolism of unsubstituted PAH (Pothuluri & Cerniglia, 1994).

Yuan *et al.* (2000) carried out aerobic biodegradation of several polyaromatic hydrocarbons including acenaphthene, phenanthrene, pyrene, fluorene and anthracene. Mixed culture degradation was not evident for anthracene and fluorene but was complete within 1.5, 10 and 12 days for phenanthrene, acenaphthene and pyrene respectively.

The biodegradation of ^{14}C-labelled di-isopropyl naphthalene with activated sludge was investigated by Yoshida and Kojima (1978b). Biodegradation quantification was by carbon dioxide evolution, and the degradation mechanism was assessed by $^{14}CO_2$ analysis by liquid scintillant counting. It was found that biodegradation at a DIPN concentration of 0.4 ppm was higher than that for 10 ppm. In a time period of 4 weeks, 20 – 40 % of the DIPN at 10 ppm was biodegraded to $^{14}CO_2$, whilst at 0.4 ppm 80 % was lost by this means. Although this indicates that DIPN is biodegradable, the extent to which this happens is clearly a function of concentration.

The largest bioremediation study was carried out to reduce toxicity in response to the extensive coastal oil pollution resulting from the *Exxon Valdez* oil spill in

Alaska. Oil spill clean-up is fast becoming the focus of large scale hydrocarbon remediation efforts, with further attempts at beach cleansing by biological means being made following the Sea Empress oil spill in Milford Haven, Wales in February 1996. It has been shown however that PAH degradation rate in contaminated soil is increased when carried out in a bioreactor as compared to *in-situ* (Rittman & Johnson, 1989, as cited in Wilson & Jones, 1993). It is also presently the case that bioremediation in treatment plants is used for the 'end-of-pipe' clean-up mechanism for most petroleum refinery wastewaters in the United States (Stringfellow & Alvarez-Cohen, 1999).

2.5.4 Surfactants

Various studies have indicated that rapid and extensive biodegradation of commercial surfactants at low concentrations occurs readily under aerobic conditions (van Gunkel, 1996; Zhang *et al.*, 1999). Margesin & Schinner (1998) investigated the bioremediation of wastewater contaminated with anionic surfactants at low temperatures with the use of bioaugmentation. The study found that biological stimulation did not affect surfactant biodegradation in the first 21 days, with 25 % of the original concentration remaining after 35 days at 10 °C regardless of the bacterial source used. The authors did state that for complete degradation of surfactants to be accomplished, a mixed microbial culture would be necessary.

Zhang and co-workers (1999) investigated the aerobic biodegradation by activated sludge bacteria of four commercial surfactant mixes at a concentration range of around 500 mg/L, which is higher than that normally considered, determining if concentration affects the biodegradation pattern. Significant degradation was evident for two of the surfactants, in another case incomplete biodegradation occurred, with a final degradation of only 65 %, and one surfactant showed no degradative losses at all.

Schröder *at al.* (1999) assessed the elimination efficiency of certain surfactants passing through the wastewater treatment process. Sampling was carried out before and after the works, followed by solid phase extraction and HPLC analysis. The study showed that the difference in elimination extent of the four

surfactants (linear alkylbenzene sulfonate - LAS, alcohol sulphonate - AS, alcohol ether sulphate – AES, and secondary alkane sulphate - SAS) were very low (99.70 % for LAS as opposed to 99.99 % for AES). All cases indicated a high degree of removal by both biodegradation and adsorption onto sludge.

Biodegradation of the primary metabolites of alkyl phenol ethoxylates was investigated by Staples and co-workers (1999) using the OECD method 301B. Biodegradation of nonylphenol ether carboxylates, octylphenol ether carboxylates and nonylphenol by aerobic microorganisms was shown to occur under these conditions, thus suggesting that they should not persist in the environment. The most recalcitrant of the compounds, nonylphenol, was shown to have a half-life of 20 days.

Adams *et al.* (1996, as cited in Kitis *et al.*, 1999), stated that nonylphenol ethoxylates are biorecalcitrant or partially biodegradable. Kitis and co-workers (1999) attempted to improve biodegradability of nonylphenol ethoxylates by using a Fenton's Reagent pre-treatment step. This was indeed achieved, with further enhanced biodegradation occurring at increased oxidant dosages.

A further study by Manzano *et al.* (1999) investigated the effect of temperature on NPE biodegradation in river water using the River Die-Away Test mentioned in Section 2.4. Experimental temperatures not only affect the degradation rates but also the lag times prior to the onset of degradation. At 7 °C, mineralisation reached 30 % in 30 days, whereas the figure was closer to 70 % at 25 °C. Biodegradation of NPEs did occur however, as indicated by residual concentration analysis by HPLC. Despite this, total mineralisation was not evident however due to the persistence of degradation intermediates. This increase in degradation metabolites was also indicated by Plomley *et al.* (1999), who noted a decrease in the higher NPE homologues, but an increase in NPE – NPE_4 following wastewater treatment. These finding are also consistent with the work by Mann & Boddy (2000) following biodegradation of NPEs by microflora in lake water.

2.5.5 Liquid scintillant cocktails

The studies mentioned above mainly deal with biodegradation of individual compounds rather than the more realistic environmental problem of mixed constituents for end-of-pipe treatment, as in the case of liquid scintillant cocktail waste. It is this aspect that requires extensive further work as it is highly likely that different biodegradation kinetics will occur when other compounds are present in the effluent, such as those found in the study by Yuan and co-workers (2000). This group investigated the biodegradation rates of individual PAH in a mixture of 5 different types. It was shown that the presence of other analytes enhanced the degradation rates of fluorene and anthracene, but negatively influenced the rates for acenaphthene, phenanthrene and pyrene.

The principle of activated sludge (AS) degradation cannot only be applied to the waste disposal of organic components but also to the biosorption of heavy metals (Battistoni *et al.*, 1993). Battistoni and co-authors found that heavy metal uptake is not only a function of the element, but also the preferred adsorption site. It is with the heavy metal adsorption capacity of AS in mind that Koyama and Nishimaki (1997) investigated the behaviour of three short-lived radionuclides, ^{134}Cs, ^{57}Co and ^{85}Sr in radioactive liquid waste. Removal processes did not involve actual biodegradation, but rather adsorption and desorption mecahnisms. Laboratory experiments under optimised degradation conditions (aeration, 20 ^{o}C and use of artificial sludge with a high biological activity) were carried out. The results showed that radionuclides, particularly ^{57}Co, can indeed be removed by AS, with the degree of uptake increasing with contact time under aeration. Removal of the ^{134}Cs and ^{85}Sr also occurred, however time scales involved were greater than for ^{57}Co. This study suggests that radionuclide removal during AS biodegradation of the organic fractions in waste scintillant cocktails may be possible.

A study was carried out by Severn Trent Laboratories (1989) into the biodegradation potential of the liquid scintillant cocktail Optiphase Hi-Safe II, which has DIPN as its primary solvent. The study was carried out according to the ISO 7827 method for the biodegradability of a test compound, where dissolved organic carbon (DOC) is assessed at the start, end and at regular

intervals to reflect analyte loss. The experiment was carried out in the dark, at a temperature of 22 - 24 °C, with a bacterial innoculum of fresh activated sludge and liquid scintillant loading of 0.005 %. This concentration was chosen to ensure an initial organic carbon level of the medium of 27.5 ± 1 mg/L, which is within the range required for the test. Optiphase Hi-Safe II was concluded to be readily biodegradable under these conditions and at these concentrations.

Klein and Gershey (1990) investigated the potential for biodegradation and toxicity of a variety of LS cocktails to the microorganisms in a secondary wastewater treatment plant (WWTP). The study started with the premise that liquid scintillant cocktails containing BAB, DIPN and phenylxylylethane were biodegradable, as advertised, whilst those containing xylene or trimethylbenzene were not. Fresh activated sludge was used, together with a nutrient medium, distilled water and 100 ppm liquid scintillant cocktail as a carbon source. Aerobic incubation on a rotary shaker, in the dark, and at room temperature was carried out, with sampling on days 0, 1, 3, 5 and 7. Analysis of the samples by gas chromatography followed solvent extraction.

The authors concluded that at cocktail concentrations from 0.005 – 0.020 %, biodegradation was a simple linear function of bacterial density, with a negative correlation with initial cocktail concentration. Biodegradation also varied depending on the primary solvent present in the cocktail. It was shown that phenylxylylethane has the shortest half-life, with LAB following, and DIPN being the most recalcitrant. The xylene and trimethylbenzene solvents were primarily lost through volatilisation and therefore could not be compared with the others. They questioned whether di-isopropyl naphthalene could actually be classed as biodegradable. In addition they concluded that under *in-situ* WWTP conditions there is a likelihood that biodegradation will not be as extensive as that found in the laboratory study. The authors suggested that an alternative to sewer disposal would be on-site pre-treatment, such as bioremediation, under optimised conditions, giving the operator more control over final composition of the effluent.

These studies indicate that:

- both degradation of the organic fraction and removal of the radionuclides appear feasible and require further investigation,
- the partial concentration of the radionuclides from the liquid scintillant cocktail into a biological matrix will allow for both transfer of the radionuclides into a more environmentally acceptable matrix and a corresponding reduction in volume of radioactive waste for disposal, and
- the use of bioreactors, and the scaling up of the process in other biodegradation studies have shown to be effective (Henner *at al.*, 1997), and therefore may also be applicable to this problem.

2.6 Degradation theory

2.6.1 Lag/adaptational phase

At the start of a biodegradation experiment there is usually a time period required prior to the onset of mineralisation. This allows for stabilisation of the system and adaptation of the biomass to the substrate and the environmental conditions present. This is known as the lag phase (Quiroga *et al.*, 1999), occurring in most new biological systems, and is characterised by a period with no analyte concentration change. In some cases, as shown by Collins & Daugulis (1999), there is a slight initial decrease in analyte concentration followed by the lag phase. These authors attributed this to the presence of a small supply of enzymes capable of biodegradation, which when exhausted caused the onset of the lag period during which further amounts of enzymes were synthesised.

The lag phase can be a function of many factors, either having a combined or isolated effect. It may involve the adaptation of a microbial strain to degradation of the substrate, or the natural selection and population growth to sufficiently high viable cell levels to survive and affect substrate concentration. Factors affecting lag phase duration include:

- availability of the enzyme required for genetic adaptation and corresponding synthesis time (Lu & Hegemann, 1998; Manzano *et al.*, 1998),

- time taken for the physical removal of some of the toxic fractions of the pollutant mix (Anderson *et al.*, 1974 as cited in Larson *et al.*, 1979; Rycroft *et al.*,1994),
- time taken for the physical reduction in the overall substrate concentration (Lu & Hegemann, 1998; Manzano *et al.*, 1998)
- analyte type (Susarla *et al.*, 1998)
- other analytes present where sequential utilisation of the compounds will result in an extended lag period for those substances that are not preferentially metabolised (Walker *et al.*, 1976).

As a result of these factors, lag phases vary greatly in length. Yuan *et al.* (2000) showed a mixed culture lag period of 3-5 h preceding a total phenanthrene degradation time of 28 h. Potter *et al.* (1999a) had a lag period of 0–12 days with complete primary degradation time of 4–24 days for nonylphenol surfactants in estuarine water samples.

The lag time of a population can be reduced by several methods, including bioaugmentation, the addition of pre-adapted microorganisms. This technique has been utilised by many biodegradation studies, including Espeche *et al.* (1994) who successfully eliminated the lag phase altogether. Zaidi *et al.* (1996) showed that the innoculum size did not effect lag phases at high *p*-nitrophenol concentrations, but a suitable microbial species could be used to shorten lag phases at low pollutant concentrations. Cross acclimation, the simultaneous adaptation of a microbial population to several substrates, can also minimise the time between first exposure to a substrate and the onset of biodegradation (Liu & Suflita, 1993). Žgajnar Gotvajn & Zagorc-Končan (1999) stated that in a nutrient limited environment, nutrient addition can in fact be more beneficial in decreasing lag times than bioaugmentation, and Manzano *et al.* (1999) suggested that temperature could also play an important role in influencing this period, with decreases in temperature resulting in increasingly high lag periods.

It must be noted that it is difficult to carry out comparisons between studies as most are conducted under different environmental conditions, and therefore lag

times are difficult to predict, potentially causing problems for the use of microbial methods for emergency pollutant remediation.

2.6.2 Abiotic losses

On monitoring biodegradation it is essential to differentiate between and quantify the relative contributions of biotic losses, that is to say 'true' biodegradation, and abiotic losses, 'apparent' biodegradation. Biotic losses are solely due to biological metabolism of a substrate, whereas abiotic losses can include such mechanisms as volatilisation, photo-oxidation and sorption onto particulate matter. It must be noted however that unlike photo-oxidation and biological processes (Fusey & Oudot, 1984), volatilisation and sorption have no direct effect on the chemical structure of the pollutants, only acting to transfer them to another medium. Abiotic losses are the primary mechanism by which analytes are changed or removed during the lag phase of an experiment.

A simple means by which to characterise biotic and abiotic losses is to determine compound loss with time both in the presence of the microbial source and using the same microbial source but deactivating it by some means such as autoclaving. This ensures that all other parameters between the two microbial sources are the same, with the biotic factor being excluded for one of the experiments. This approach was used by Potter *et al.* (1999b) who found that despite thorough autoclaving of the sludge used as a bacterial source, biomass concentrations in both the autoclaved and untreated sludge were comparable. Despite dismissing this sterilisation technique as being ineffective, it may simply have resulted from incomplete sterilisation due to the large volumes used and its high water content. Stagg *et al.* (1996) used mercuric chloride as an inhibitor of microbial activity as an alternative to autoclaving. They found that substantial analyte losses in these controls could be attributed either to the mercuric chloride being ineffective or to the impact of abiotic mechanisms. Kirby (1994) also used mercuric chloride, and considered the anti-microbial action to be effective and therefore suitable for use in determining abiotic losses.

Sometimes a component of the pollutant mix that is resistant to biodegradation is used to monitor abiotic losses. Hopane is often used for this purpose in crude

oil biodegradation studies, as by Atlas (1995), Bragg *et al.* (1994) and Venosa *et al.* (1996), whilst Pritchard & Costa (1991) used pristane and phytane. As for internal standards, control compounds are generally used to test compounds that have similar physical and chemical behaviours, on the premise that most abiotic processes occurring will be comparable between the two.

2.6.2.1 Sorption

Sorption is a general term used for the processes of adsorption, absorption, chemisorption and persorption (Larousse, 1995). Natural biodegradation rates and extents may be influenced by the sorption and desorption of pollutants on particulate matter (Burgos *et al.*, 1999; Shuttleworth & Cerniglia, 1995). PAH have limited solubilities and strong sorption tendencies (Boonchan *et al.*, 1998; Leblond *et al.*, 2001; Shiaris, 1989; Woodhead *et al.*, 1999), particularly to the organic fraction of soil and sediments (Kordel *et al.*, 1997, as cited in Ressler *et al.*, 1999; Stringfellow & Alvarez-Cohen, 1999). Studies have shown that degradation rates, particularly of two- and three-ring PAH compounds (Wilson & Jones, 1993), can be controlled by their dissolution rates from soil organic matter (Mulder *et al.*, 1998; Shuttleworth & Cerniglia, 1995).

Many endocrine-disrupting substances, such as nonylphenol ethoxylates, have also been shown to be strongly absorptive to particulates and will therefore be readily removed from solution by this method (Bennie *et al.*, 1997; Field & Reed, 1999; Lye *et al.*, 1999; Shang *et al.*, 1999b). This is in contrast to another study into the biodegradability of nonionic surfactants which concluded that these compounds have a low potential for sorptive losses onto particulates (Kitis *et al.*, 1999). The contrasting conclusions could be as a result of selection of different ethoxylate (EO) chain lengths and concentrations, as Kibbey & Hayes (2000) showed that at low concentrations sorbed surfactants were dominated by high-EO components, whereas with increasing concentrations low EO-components were preferentially sorbed. Linear alkyl benzenes are also considered to be high-adsorptive substances (Binetti *et al.*, 2000), although strong sorption does not necessarily occur (Westall *et al.*, 1999).

Bonding onto organic matter by sorption makes the compound less available as a carbon source and thus microorganisms will preferentially use other available carbon sources (Eriksson *et al.*, 2000; Prince, 1993; Ressler *et al.*, 1999; Singleton, 1994). Initial adsorption is fast, with a subsequent decrease in rate and adsorption equilibrium being attained eventually (Hwu *et al.*, 1998). Prolonged sorption results in increased resistance to degradation and extraction (Calderbank, 1989, as cited in Burgos *et al.*, 1999), and can be onto dead or living matter. Ressler and co-workers (1999) even suggested that some PAH metabolites bound irreversibly to particulate organic matter, and were therefore not only unavailable for biodegradation and could also not be desorbed by solvent extraction. However Stringfellow & Alvarez-Cohen (1999) questioned whether adsorption could be considered a true loss, or simply a temporary removal from solution allowing for solvent extraction and analysis.

Bioavailability of substrates sorbed onto organic substances will vary depending on the sorption mechanism, particle size, pH and sorption site. Oxidative covalent bonding to humic substances is less readily broken than physiosorption to an external soil surface. Physiosorbed compounds are also more easily desorbed in the presence of bacteria acting as a 'release factor' (Burgos *et al.*, 1999). Gao *et al.* (1998) investigated the sorption of pesticides onto sediment and concluded that organic carbon content and particle size play an important role, with a decrease in organic content greatly reducing sorption capacity. In addition these authors found that increasing the pH from 2 to 10 resulted in decreased sorption and increased desorption of the pesticides.

The primary site of sorption in this study would be onto the particulates and biomass of the activated sludge used. A study by Naylor (1995) showed that there is only a very small proportion of combined nonylphenol ethoxylates present in digested sludge (1.7 % maximum of the total present in wastewater treatment plant water), suggesting that this is not a major sink of the pollutants. However, Ejlertsson *et al.* (1999) and Marcomini *et al.* (1990) showed that nonylphenol ethoxylates with one or two ethoxylate groups were less hydrophilic and therefore more readily sorbed to sludge constituents and organic matter. Brubaker and Stroo (1992) showed that the four- and five-ringed

PAH were more readily sorbed onto particulates, and Koyama & Nishimaki (1997) also found high levels of radionuclide removal by the activated sludge process. These studies show that sorption capacity is very compound specific.

2.6.2.2 Volatilisation

Volatilisation is also known as vaporisation, and involves the conversion of a liquid or solid into a vapour. It will primarily occur with low molecular weight compounds and should decrease with time (Floodgate, 1984) in an open system. Al-Saadi (1991, as cited in Al-Hadhrami *et al.*, 1997) attributed C_{12} alkane losses to evaporation. It is also frequently noted that oil spill biodegradation does not commence until the lighter crude oil fractions have been volatilised (Dart & Stretton, 1980).

Addison *et al.* (1983, as cited in Addison, 1983) suggested that KMC-A, a dielectric fluid whose primary solvent is di-isopropyl naphthalene (boiling point = 290 – 299 °C), would be more likely to volatilise and partition into the atmosphere rather than be adsorbed to particulate material. Gledhill *et al.* (1991, as cited in Binetti *et al.*, 2000) suggested that linear alkyl benzenes would undergo 'moderate volatilisation' from the aqueous medium. As for sorption, volatilisation does not play a significant role in determining the fate of PAH compounds with more than three rings (Wilson & Jones, 1993), however, it will have an impact on those with fewer aromatic rings. A study by Kitis *et al* (1999) showed that due to their low vapour pressures, non-ionic surfactants had a low volatilisation potential.

2.6.2.3 Photo-oxidation

In addition to volatilisation, degradation of crude oil has been shown to occur by photo-oxidation. This is also known as photochemical breakdown and is a chemical breakdown as a result of radiation e.g. a light-catalysed reaction. In this case photo-oxidation occurs preferentially at the tertiary carbons, often removing methyl- groups from branched alkanes (Bartha & Atlas, 1987). The effective wavelengths are shorter than 400 nm, with products generally being more soluble than the parent compounds and therefore more available for microbial attack (Floodgate, 1984).

Alkyl-phenol ethoxylates have also been shown to degrade by photochemical breakdown (Ahel *et al.*, 1994, as cited in Davi & Gnudi, 1999). Whereas Gledhill *et al.* (1991, as cited in Binetti *et al.*, 2000) stated that on exposing an acetonitrile solution of linear alkyl benzenes to sunlight there was no evidence of photo-oxidative losses. The literature is therefore inconclusive regarding this analyte.

2.6.3 Biodegradation mechanisms and products

In addition to abiotic losses, biotic losses can also have an impact on compound concentration. The use of biodegradation principally implies the utilisation of microbes to enhance biotic losses. The type and distribution of degradation products depends on the enzymes and environmental conditions used. Dependency on enzyme type is not well understood but it has been suggested that different enzymes will favour different catalytic mechanisms and thus result in different metabolic products (Aitken *et al.*, 1994). It is also generally accepted that different microbes will use different degradation pathways, often producing the same, but also having the potential for variable products. For example it is well documented that the oxidation of unsubstituted polyaromatic hydrocarbons (PAH) by bacteria requires the use of dioxygenase, whereas both fungi and mammals use monooxygenase. In the case of fungi this metabolism is via the cytochrome P-450 monooxygenase (Cerniglia, 1984; Pothuluri & Cerniglia, 1994; Wilson & Jones, 1993). Other factors, such as environmental pH, can also affect the resultant products from a given enzyme/substrate combination. Aitken *et al.* (1994) suggested that this is possibly due to changes in intrinsic enzyme rate constants and elementary reaction rates, and effects on the stability of metabolites.

When considering the degradation products, complete biodegradation should ultimately produce carbon dioxide, water and biomass. Given ideal conditions, the necessary enzymes and sufficient time, this will indeed occur, however, often the metabolism does not go to completion as a direct result of non-ideal degradation conditions, for example the absence of specific microbial species required to complete the biodegradation process. Several metabolic

intermediates are known to be more toxic than the original pollutant (Aitken *et al.*, 1994; Prince, 1993). This can cause great environmental concern, and provides ammunition for the opponents of biodegradation.

2.6.3.1 Aliphatic hydrocarbons

These compounds are primarily metabolised by mixed function monooxygenases acting on the terminal carbon atom to produce primary alcohols (Bartha & Atlas, 1987; Gibson, 1980; Prince, 1993) (Figure 2.8). In some cases microbial attack takes place on the subterminal carbon atom, in which case a secondary alcohol is produced (Gibson, 1980; Prince, 1993) (Figure 2.9). The molecular oxygen used in the process is crucial as an electron acceptor (Al-Hadhrami *et al.*, 1997). Two systems are able to carry out the process, namely the P-450 system using iron protein and NADPH, and the rubredoxin system solely using NADH (Bartha & Atlas, 1987).

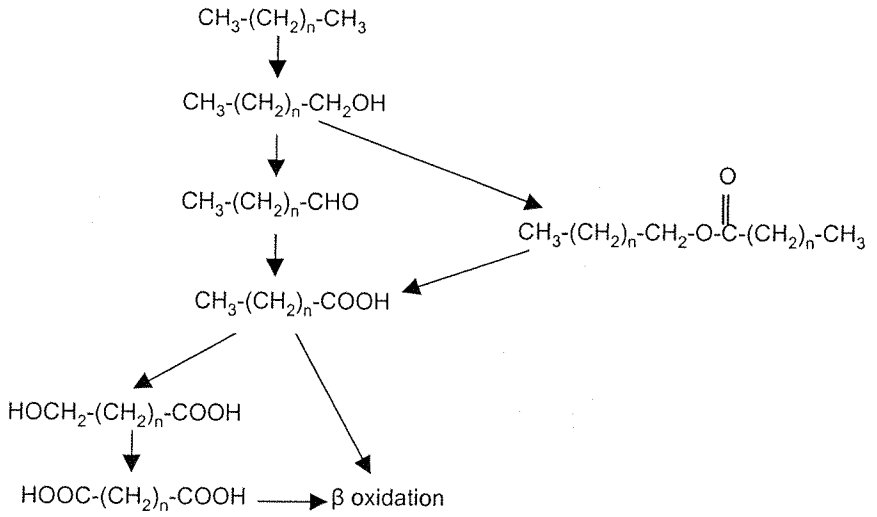


Figure 2.8 Terminal carbon atom degradation pathway (Source: Britton, 1984)

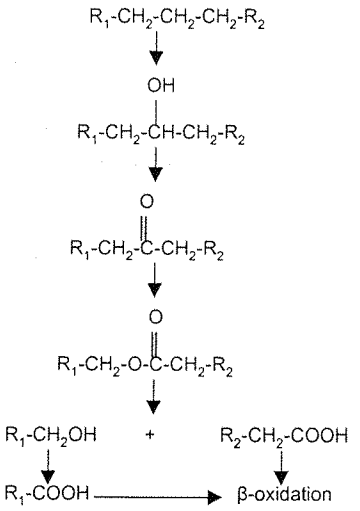


Figure 2.9 Subterminal carbon atom alkane degradation pathway (Source: Britton, 1984) R = H or alkyl group

Primary alcohols are further metabolised to form aldehydes using NADP (Britton, 1984), and then fatty acids which subsequently take part in the Krebs cycle (Al-Hadhrami *et al.*, 1997; Britton, 1984; Dart & Stretton, 1980). Secondary alcohols from subterminal carbon atom oxidation proceed to form ketones, esters (generally formate or acetate ester), formic or acetic acid, primary alcohols, aldehydes and finally fatty acids (Bartha & Atlas, 1987).

2.6.3.2 Alkenes

These structures undergo metabolism either at the saturated terminal carbon atom, or alternatively the double bond is oxidised forming epoxy compounds. Next one hydroxyl group is transformed to produce a carboxyl group, followed by cleavage to form fatty acids and primary alcohols (Bartha & Atlas, 1987) (Figure 2.10).

2.6.3.3 Alkylbenzenes

There is some dispute over the method by which alkylbenzenes like toluene are biodegraded. Smith & Ratledge (1989, cited in Prince, 1993) stated that ring fission occurred first, followed by side chain oxidation. However, Cerniglia (1984) and Wrenn (1998) suggested that primary degradation took place on the aliphatic side chain by the action of monooxygenases to produce benzyl alcohol which are further dehydrogenated to form benzoic acid and eventually, through

oxidation of the aromatic ring, catechol is formed. Catechol is the starting substrate for ring fission. It is likely that the two degradation stages are controlled by different enzymes, possibly in the same organism, and possibly even simultaneously (Prince, 1993).

Several studies have been carried out on the biodegradation of nonylphenol ethoxylates. Ding & Tzing (1998) and Di Corcia *et al.* (1998a, 1998b) suggest that there are two separate routes that can be taken. The first mechanism being the sequential shortening of the ethoxylate chain to produce alkylphenols with less than three ethoxylate units, and the second involving carboxylation of the terminal ethoxylate unit to produce alkylphenol carboxylates. Most authors combine these two with a resultant mechanism which utilises ether cleavage (Di Corcia *et al.*, 1998a) of the ethoxylate chain to produce ethylene glycol and nonylphenol ethoxylates with one or two ethoxylate units (Argese *et al.*, 1994; Bennie *et al.*, 1997; Castillo *et al.*, 1999; Ejlertsson *et al.*, 1999; Manzano *et al.*, 1999; Naylor, 1995). Further oxidation of the terminal ethoxylate unit results in low molecular mass carboxylated nonylphenols (Argese *et al.*, 1994; Di Corcia *et al.*, 1994, 1998a; Ejlertsson *et al.*, 1999; Lye *et al.*, 1999; Maki *et al.*,1996, 1998; Mann & Boddy, 2000; Potter *et al.*, 1999a; Scullion *et al.*, 1996; Shang *et al.*, 1999b). In comparison to the parent compounds, these products are relatively toxic (Argese *et al.*, 1994) and non-biodegradable (Manzano *et al.*, 1998), but can be lost by sorption (Ejlertsson *et al.*,1999). Further oxidation to form carbon dioxide and water is slow, but possible (Lye *et al.*, 1999; Potter *et al.*, 1999a), although Maki *et al.* (1998) found that nonylphenol ethoxylate derivatives of low molecular mass disappeared within 24 hours.

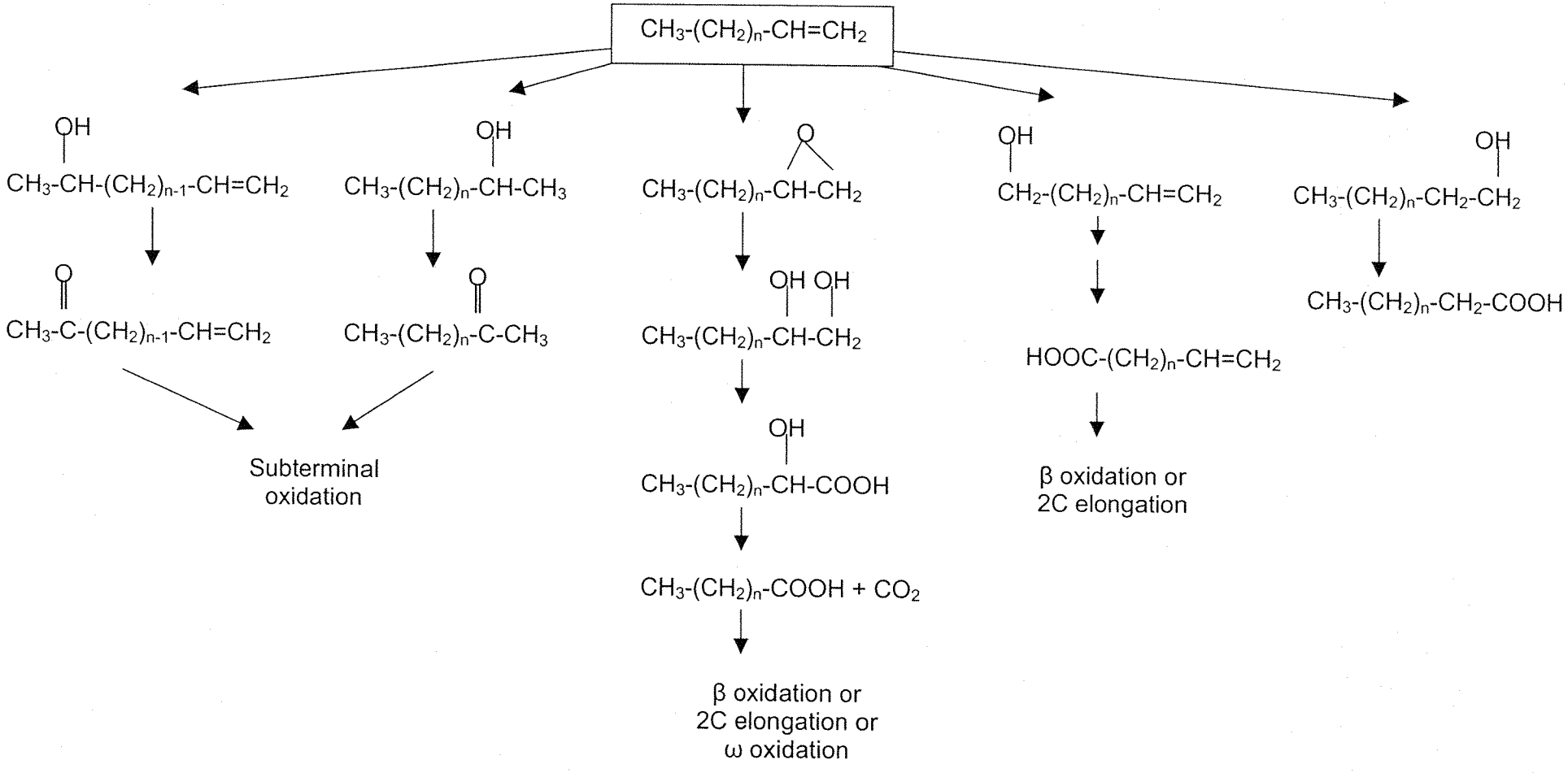


Figure 2.10 Alkene degradation pathways (Source: Britton, 1984)

A few studies have been conducted regarding the metabolism of di-phenyl oxazole (PPO). Mutch and co-workers (1985) were unable to identify the metabolites produced by PPO degradation. They did conclude however that in human liver microsomes, metabolism is likely to be dependent on more than two separate cytochrome isozymes, whereas in rat liver microsomes a single major form of cytochrome P-450 is used. This latter deduction was backed up by a study on the metabolism of PPO by Ahokas *et al.* (1987), who also found that three main metabolites were formed, namely: 5-(*o*-hydroxyphenyl)-2-phenyloxazole, 2-(*p*-hydroxyphenyl)-5-phenyloxazole, and 5-(*p*-hydroxypentyl)-2-phenyloxazole as the primary product.

2.6.3.4 Polyaromatic Hydrocarbons

As mentioned previously, bacteria, fungi and mammals are capable of metabolising PAH. The bacterial degradation route involves the use of *cis*-diol, and NAD^+ to produce $NADH_2$, whilst the fungal and mammalian pathway involves *trans*-diol and the conversion of $NADP^+$ to $NADPH_2$ (Gibson, 1980). As for all other analytes, degradation of an individual or suite of compounds is enhanced if a mixture rather than a single isolate is present. This is due to some of the species degrading the PAH metabolites, thus reducing chances of toxicity accumulation, and facilitating total PAH removal (Leblond *et al.*, 2001; Sylvestre & Sondossi, 1994). Many fungi that are incapable of using PAH as a carbon and energy source still participate in the oxidation of these compounds by co-metabolism (Gibson, 1980).

Ring cleavage is necessary for complete metabolism of PAH. It can only occur if there are a minimum of two hydroxyl groups present (Gibson, 1980), and requires destabilisation of the benzene ring to make it more reactive. This is achieved by oxidation, resulting in the formation of benzene dihydrodiol and subsequently catechol. As well as oxygen and dioxygenase enzymes, an electron donating co-substrate like $NADH^+$ is required (Wrenn, 1998). The process can occur by two different mechanisms, namely the *ortho-* and *meta-*pathways (Figure 2.11 and 2.12). *Ortho-* cleavage involves splitting of the ring between two carbon atoms with hydroxyl groups, whilst *meta-* cleavage is the ring split between an unsubstituted carbon atom and one which is hydroxylated

(Wrenn, 1998). Both of these have the same basic requirements of enzymes, oxygen and co-substrates, and the same products of acetate and succinate, or possibly pyruvate and acetaldehyde which are readily converted to the former two compounds (Wrenn, 1998).

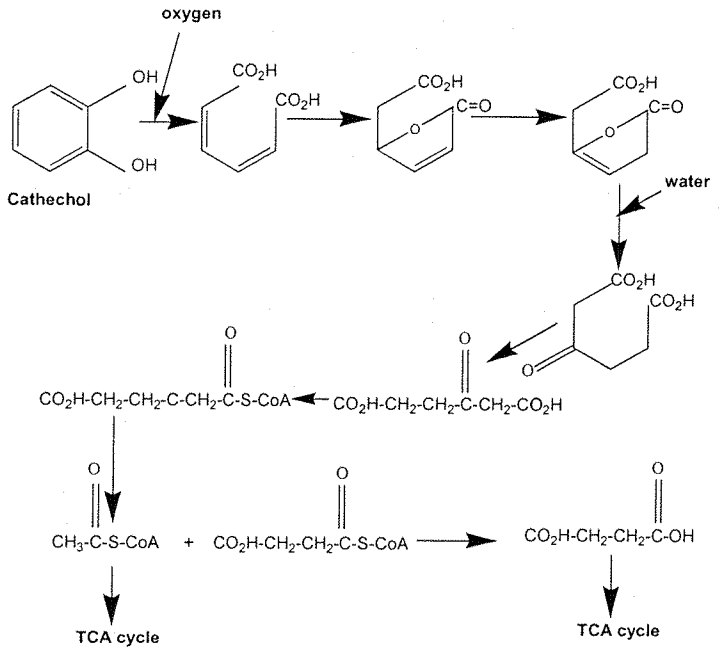


Figure 2.11 ***Ortho*- cleavage of catechol following initial oxidation** (Source: Wrenn, 1998)

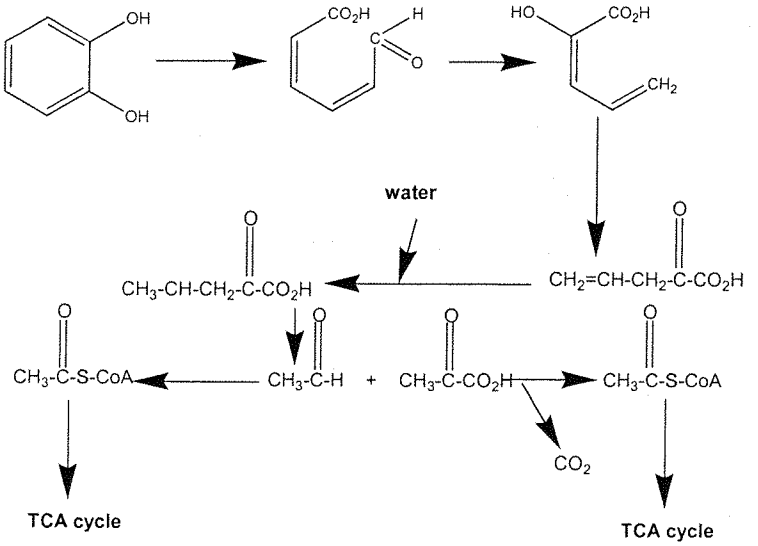


Figure 2.12 ***Meta*- cleavage of catechol following initial oxidation** (Source: Wrenn, 1998)

As shown for benzene degradation in Figures 2.11 and 2.12, naphthalene is degraded by successive opening of the aromatic rings (Bartha & Atlas, 1987). It doesn't participate directly in biologically mediated or surface catalysed oxidative coupling reactions, but once transformed into a hydroxylated intermediate like salicylate, oxidation is possible (Burgos *et al.*, 1999). A study by Bauer & Capone (1988) showed that all PAH with more than two rings degraded through naphthalene.

As far as di-isopropyl naphthalene is concerned, both the side chains and the naphthalene nucleus can be biodegraded (Yoshida & Kojima, 1978b), with aliphatic side chain oxidation resulting in the formation of acids, alcohols and their conjugates (Addison, 1983) (Figure 2.13). It must be noted that as mentioned for alkane degradation, the metabolites produced, such as oxidised naphthalenes, may be more toxic than the parent compounds (Höke & Zellerhoff, 1998; Shuttleworth & Cerniglia, 1995), and therefore the use of a mixed culture may be necessary to minimise these risks.

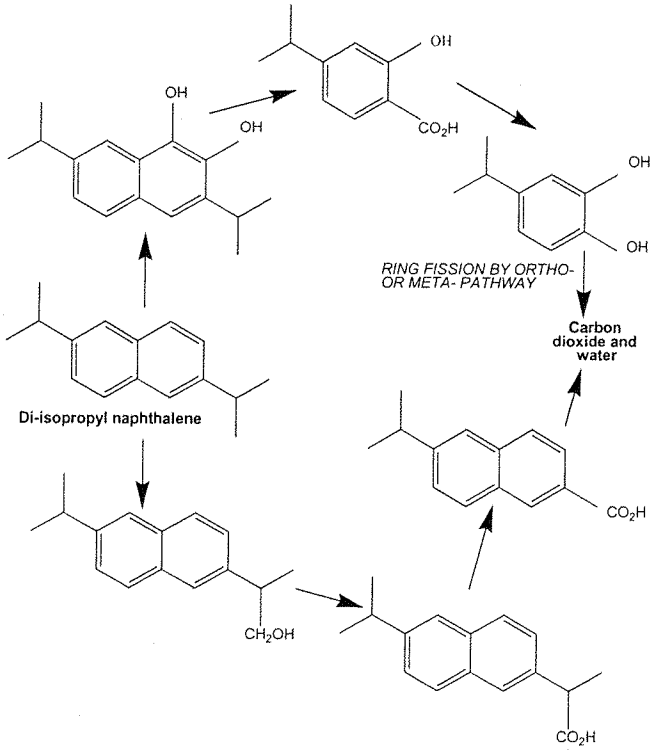


Figure 2.13 Summary of the major biodegradation pathways of 2,6-di-isopropyl naphthalene (Source: Yoshida & Kojima, 1978b)

2.6.4 Biodegradation preference

The order of preferential degradation is primarily a function of compound structure and microbial species present. The range of hydrocarbons utilised for growth by individual organisms is usually limited (Britton, 1984). However as mentioned previously, co-metabolism, where the energy for growth is derived from a compound other than that being metabolised at that point, is possible, particularly for large structures like high molecular weight aromatics and polychlorinated biphenyls (Liu & Suflita, 1993). When co-metabolism does occur it is generally within a structural grouping as aromatic degraders are incapable of biodegrading aliphatic molecules and *vice versa* (Foght *et al.*, 1990 as cited in Prince, 1993). In addition, the combined toxicities of several compounds may act to inhibit the acclimation and adaptation ability of microbes, thus limiting metabolism (Bauer & Capone, 1988).

A generally accepted order of decreasing susceptibility to biodegradation is as follows:

- alkanes,
- branched alkanes,
- polyaromatic hydrocarbons,
- alicyclic alkanes

(Atlas, 1981, 1991; Dorn *et al.*, 1993; Dugenest *et al.*, 1999; Floodgate, 1984; Fusey & Oudot, 1984; Hambrick III *et al.*, 1980; Jobson *et al.*, 1974; Leahy & Colwell, 1990; Prince, 1993; Pritchard & Costa, 1991; Tagger *et al.*, 1983; Wrenn, 1998). This incorporates the fact that polar compounds are more resistant to biodegradation than non-polar compounds (Rontani *et al.*, 1986), and parent compounds are more easily degraded than their alkylated derivatives with a substitution of up to four methyl groups (Bayona *et al.*, 1986 as cited in Prince 1993).

2.6.*4.1 Alkanes*

There is a reduced susceptibility to degradation with increased chain length (Hakstege & van Geldermalsen, 1998; Prince, 1993; Venosa *et al.*, 1993). This is particularly marked for bacteria and yeasts, but is not as evident for filamentous fungi (Atlas, 1981). It is also apparent that degradation and

assimilation do not necessarily coincide, as longer chained alkanes, like nonane, are more readily assimilated than methane and ethane. This is possibly as a result of the shorter chain lengths being more soluble in the aqueous phase and therefore have a greater toxicity (Britton, 1984). On balancing out the theoretical susceptibility and the toxicity factor, it is the C_{10}-C_{18} alkanes that are most readily attacked, particularly by bacteria (Britton, 1984). This author also stated that the presence of both double bonds and branching decreases the susceptibility to biodegradation.

2.6.4.2 Aromatic hydrocarbons

It is generally accepted that ease of biodegradation decreases with increased number of aromatic rings (Atlas, 1991; Boonchan *et al.*, 1998; Brubaker & Stroo, 1992; Hakstege & van Geldermalsen, 1998; Pothuluri & Cerniglia, 1994; Potter *et al.*, 1999b; Shuttleworth & Cerniglia, 1995; Venosa *et al.*, 1993; Walker *et al.*, 1975b; Wilson & Jones, 1993). This is likely to be a function of decreased solubility and volatility, resulting in an increased resistance (Bennett *et al.*, 1999). For PAH structures with four or more rings co-metabolism is often required (Atlas, 1981; Pothuluri & Cerniglia, 1994; Wilson & Jones, 1993).

Heitkamp & Cerniglia (1989) stated that the simultaneous presence of other PAH does not affect the extent of biodegradation. However, toluene degradation was shown to decrease in the presence of benzene in a study by Collins & Daugulis (1999). This was attributed to the same enzyme requirement for degradation of both of the aromatic structures. In addition enzyme induction for PAH metabolism actually depends upon the presence of lower molecular weight aromatics like naphthalene (Heitkamp *et al.*, 1988, as cited in Atlas, 1991).

Factors that reduce aromatic biodegradation susceptibility include:

- presence of sulphur within the structure (Walker *et al.*, 1975b),
- methyl- substitution on adjacent carbon atoms (Bayona *et al.*, 1986 as cited in Prince, 1993),
- condensed polyaromatic compounds (Floodgate, 1984),
- angular rather than linear ring arrangement (Wilson & Jones, 1993),

- halogenation (Buitrón *et al.*, 1998), and
- the incorporation of an alicyclic ring between two aromatics (Walker *et al.*, 1975a, 1975b).

Di-isopropyl naphthalene is considered to be readily biodegradable (Addison, 1983). The alkyl substituents on the aromatic rings are preferentially oxidised over the naphthalene structure itself (Höke & Zellerhoff, 1998). The presence of this substitution results in a decrease in sites of microbial attack and therefore reduces the biodegradation potential (Leblond *et al.*, 2001). However, substituents on opposite sides of the molecule, as in the case of 2,6- and 2,7-di-isopropyl naphthalene, are considered to increase susceptibility to biodegradation (Bayona *et al.*, 1986 as cited in Prince, 1993). In addition, the butyl side chains and high levels of alkyl substitution on the aromatic moiety reduces the likelihood of producing reactive and harmful intermediates, as in the case of naphthalene degradation (Höke & Zellerhoff, 1998).

Several studies have been conducted specifically on the biodegradability of nonylphenol ethoxylates. Marcomini *et al.* (1990) and Dorn *et al.* (1993) found that those compounds with longer ethoxylate chains degraded more readily due a decreased toxicity, whereas toxicity levels were increased with increased aliphatic chain length, thus reducing susceptibility (Dorn *et al.*, 1993). In both cases however, it is the ethoxylate chain, rather than the alkyl section that is most readily metabolised (Sherrard *et al.*, 1996), particularly if the alkyl section is branched (Dorn *et al.*, 1993; van Ginkel, 1996). Branching of either the ethoxylate or alkyl- chain reduces their biodegradation potential (Ejlertsson *et al.*, 1999), as does the addition of a further aromatic ring (Zhang *et al.*, 1999).

2.6.4.3 Cyclic Alkanes

Cyclohexane is considered to be very readily biodegradable (Bean & Perry, 1974 as cited in Gibson, 1980). There is not a simple correlation between structure and susceptibility, with the order of decreasing ease of degradation being six > one > two > three > five > four rings (Walker *et al.*, 1975a, 1975b).

2.6.5 Degradation rate

Closely associated with the degradation preference is the rate at which degradation occurs. It can logically be assumed that if a structure is readily biodegradable, as in the case of linear alkanes, then the rate will be higher than for a compound that is less readily metabolised, such as PAH. Despite a large amount of quantitative rate data present in the literature, it is difficult to compare these data as differences in methodology and environmental conditions used make this comparison invalid. This section will therefore only discuss trends and factors that influence degradation rates.

Degradation rate is controlled by many different factors including the reaction conditions and compound structure. An increase in biodegradation rate may be as a result of:

- increased hydroxyl radicals e.g. by the addition of Fenton's reagent (Martens & Frankenberg Jr., 1995),
- surfactant addition (Jahan *et al.*, 1999; Mulligan *et al.*, 1999; Shuttleworth & Cerniglia, 1995),
- small or no substituent groups (Leblond *et al.*, 2001),
- optimised pH (Hambrick III *et al.*, 1980),
- starting concentrations above the threshold, allowing for acclimation and adaptation (Bauer & Capone, 1988; Kerr & Capone, 1988; Shuttleworth & Cerniglia, 1995), but below the toxic level (Abd-Allah & Srorr, 1998; Atlas, 1991; Fusey & Oudot, 1984),
- thorough mixing of the solution (Jee, 1998),
- presence of a mixed culture (Atlas, 1977),
- microbial concentration in the case of a mixed culture (Baughman *et al.*, 1980, as cited in Spain & van Veld, 1983), and/or
- the presence of the necessary nutrients and microbes (Atlas, 1995; Margesin & Schinner, 1998; Pothuluri & Cerniglia, 1994; Žgajnar Gotvajn & Zagorc-Končan, 1999).

Surfactant addition works on the basis of micelle formation, increasing solubilisation and surface area (Boehm & Fiest, 1982; Boonchan *et al.*, 1998; Choori *et al.*, 1998; Leahy & Colwell, 1990; Rouse *et al.*, 1994). Hydrocarbons

present in the micelles are not directly available for biodegradation but must first be dissolved in the aqueous phase (Mulder *et al.*, 1998). Following solubilisation a several fold increase in degradation rate is apparent (Zhang & Miller, 1992 as cited in Page *et al.*, 1999). Several authors have shown a marked increase in degradation rate following surfactant addition, particularly for five- to seven- ring PAH (Rouse *et al.*, 1994; Shuttleworth & Cerniglia, 1995; Zimmerman *et al.*, 1999). Surfactant addition has also been found to reduce biodegradation rate on occasion. This unexpected result was found in a study by Abd-Allah & Srorr (1998) who used Triton-X-100, and was possibly as a result of the surfactant itself being used as the primary carbon source (Goudar *et al.*, 1999), or alternatively that high concentrations used resulted in a toxicity effect (Mohammed *et al.*, 1996).

Overall the primary determinant of degradation rate is the availability of the substrate, as low solubility results in limited transport into the microbial cell and thus a decreased degradation rate (Singleton, 1994). The secondary control factor is the electronic features of the compound itself. This includes the substitutions on aromatic rings and the presence of certain key groups. For example, a hydroxyl group has little influence whereas a carboxyl group greatly enhances the rate (Okey & Stensel, 1996). Regardless of these variables however, highest rates will occur at the start of any experiment following the lag phase (Espeche *et al.*, 1994; Margesin & Schinner, 1998; Potter *et al.*, 1999b; Pritchard & Costa, 1991; Quiroga *et al.*, 1999; Shuttleworth & Cerniglia, 1995) when there are no nutrient limitations and there has been insufficient time for toxic metabolite build up.

Bearing these considerations in mind the general order of decreasing degradation rate is:

- linear alkanes,
- branched alkanes,
- alkenes,
- cycloalkanes,
- aromatics (Fusey & Oudot, 1984; Hambrick III *et al.*, 1980).

This is very similar to the degradation preference outlined in Section 2.6.4.

Tentative models to predict degradation rates on the basis of a compound's molecular structure have been developed. These models are termed quantitative structure-biodegradability relationships (QSBRs). One such model is that outlined by Okey & Stensel (1996), who investigated the prediction of organic compound biodegradation rates in activated sludge or comparable biological systems. These models rely on the use of proper descriptors and an extensive database, but cannot account for all environmental variables and can therefore only be seen as a complementary rather than diagnostic tool.

A study of linear alkyl benzene degradation rates showed that starting concentration played an important role in determining degradation rate. At 0.1 – 0.5 ppm, over 90 % of the original concentration was lost by biodegradation in 28 days (Gledhill *et al.*, 1991 as cited in Binetti *et al.*, 2000). However, at linear alkyl benzene concentrations above this only 65 % was removed in this time period (Binetti *et al.*, 2000).

Due to their regular presence in the environment, many studies have been conducted on the biodegradation rate of PAH. Rates have been shown to decrease with increasing molecular mass (Shiaris, 1989), ring numbers (Brubaker & Stroo, 1992; Potter *et al.*, 1999b; Wilson & Jones, 1993), and number and position of substitutions on the aromatic ring (Susarla *et al.*, 1998).

Chapter 3 - Bioreactor set up

In this study biodegradation experiments were carried out in bioreactors. The bioreactor set up changed during the three-year span of the project as the method developed and new discoveries were made, proceeding in two distinct stages. Initial experiments utilised four bioreactors, increasing to twenty-six for later experiments. This chapter aims to describe some of these developments along with discussing the sampling procedure and data collection system.

3.1 Activated sludge as a suitable microbial source

Activated sludge (AS) is the solid and liquid effluent product from the activated sludge process (Hawkes, 1983). This process is used worldwide as a secondary stage in sewage treatment process and the treatment of many industrial organic effluents (Boehnke *et al.*, 1998; Hawkes, 1983; Mayhew & Stephenson, 1997). The activated sludge process involves mixing sewage effluent from the primary sedimentation tank with AS, followed by actively aerating the mixture to reduce the soluble organic content of the sewage and its biological oxygen demand (BOD). Following aeration the AS/sewage mixture (the mixed liquor) is transferred to a settling tank to separate the solid flocs from the purified liquid effluent. The liquid effluent is then discharged into a water body for further dilution, such as a river or estuary. Part of the settled solid matter is recycled into the aeration tank as a microbial source for further organic metabolism of fresh sewage, whilst the remainder is de-watered and used as fertiliser, disposed of into landfills, dumped at sea or incinerated.

3.1.1 Microbial diversity and metabolic capabilities

Contaminants frequently appear in activated sludge as a result of domestic and industrial sewer disposal. Amongst the organic examples, the most common are aromatic hydrocarbons, organochlorinated compounds, aliphatic hydrocarbons, amines, nitrosamines, phenols, esters and phthalates (Atlas, 1977; Moreda *et al.*, 1998; Schröder, 1993; Staples *et al.*, 1999). As a result of this variety of organic carbon sources, a diverse ecosystem of microorganisms develops in the sludge, resulting in the formation of flocs. The structural and functional bases of activated-sludge flocs are primarily bacteria (Hawkes, 1983) and protozoa (Singleton & Sainsbury, 1987). Bacteria commonly present include *Acinetobacter* spp., *Alcaligenes* spp. and *Sphaerotilus natans* (Mayhew &

Stephenson, 1997; Singleton & Sainsbury, 1987). Protozoa include ciliate, flagellate and amoeba species (Singleton & Sainsbury, 1987).

The use of activated sludge as a microbial source for the biodegradation of organic compounds is extensive and well documented (Buitrón *et al.*, 1998; Zagorc-Končan & Šömen, 1999). The activated-sludge flocs provide a matrix onto which some components of the waste are adsorbed. Some of the removed organic matter is immediately oxidised by the microbial content of the flocs whilst other degradable fractions are oxidised more gradually. The aerobic metabolism of organic matter by microorganisms may be represented as:

$$\underset{\text{(organic matter)}}{COHNS} + O_2 + \text{nutrients} \xrightarrow{\text{bacteria}} CO_2 + NH_3 + \underset{\text{(new bacterial cells)}}{C_5H_7NO_2} + \text{other end products}$$

Microbes possessing the genetic capability to metabolise a specific carbon source and utilise it for growth are known as the competent biomass (Magbanua *et al.*, 1998). Many microbial populations adapted to metabolise a particular substrate can simultaneously mineralise other, structurally related, carbon sources (Bartha & Atlas, 1987; Bauer & Capone, 1988; Liu & Suflita, 1993). Even if the carbon source is not used as an energy source, pollutants may still be transformed by co-metabolism (Atlas, 1981; Bartha & Atlas, 1987; Britton, 1984; Gibson, 1980; Liu & Suflita, 1993; Romero *et al.*, 1998). Spain & Van Veld (1983) defined adaptation as 'a change in the microbial community resulting in an increased transformation rate of a compound as a result of prior exposure to that compound'. Adaptational responses involve:

- the induction or stimulation of specific enzymes not present, or only present at low levels,
- selection by genetic changes resulting in new metabolic capabilities, and/or
- increase in the microbial biomass capable of catalysing a specific metabolic pathway (Leahy & Colwell, 1990; Spain *et al.*, 1980; Sylvestre & Sondossi, 1994).

Most of the adaptive systems appear only after a contact time with the specific pollutant ranging from a few minutes to several days (Minas & Gunkel, 1995; Tagger *et al.*, 1983). Time taken for adaptation to occur depends on the growth rate of the specific bacteria (Lu & Hegemann, 1998). In addition there may be a threshold contaminant concentration and microbial biomass below which no adaptation takes place (Spain *et al.*, 1980; Spain & van Veld, 1983).

The literature indicates that a mixed culture will be required for the biodegradation of complex molecules and mixtures such as liquid scintillant cocktails (Atlas, 1977, 1995; Buitrón *et al.*, 1998; Cerniglia, 1984; Dart & Stretton, 1980; Leahy & Colwell, 1990; Levinson *et al.*, 1994; Minas & Gunkel, 1995; Slater & Lovatt, 1984; van Ginkel, 1996). It is very difficult to isolate single microorganisms that have the capability to degrade complex molecules. This degradation is usually achieved only by a collection of microbes with complementary degradative properties acting either simultaneously or sequentially (Dart & Stretton, 1980; Gibson, 1980; Slater & Lovatt, 1984; van Ginkel, 1996).

The possibility of prior adaptation suggests that as the contaminants passing through the sewage treatment works are of such a varied nature, the microbial community will have at least some members that are already adapted to the use of each of the majority of these chemicals as a carbon source (Atlas, 1977). On taking this argument further, this would also suggest that the population as a whole could be further adapted to metabolise the different carbon sources in the waste being processed (Bauer & Capone, 1988; Margesin & Schinner, 1998). This should then incorporate all of the different chemicals present in the liquid scintillant cocktail that is the focus of this work.

In addition to the capacity of organic compound metabolism, the activated sludge is a very accessible and cheap source of microbes. This not only makes this option attractive from an economical perspective, but also means that the technique can be used in areas where there are technological limitations, such as in some developing countries.

3.1.2 Activated sludge use in this and other studies

Activated sludge has been used extensively to treat both domestic and industrial waste (Maszenan *et al.*, 1998). Some examples of use are in the study of:

- the biodegradation of anionic surfactants in the presence of organic contaminants (Abd-Allah & Srorr, 1998),
- biotransformation of nonylphenol ethoxylates (Di Corcia *et al.*, 1998b),
- the biodegradation intermediates of branched alcohol ethoxylate surfactants (Di Corcia *et al.*, 1998a),
- the denitrification kinetics of high nitrate concentration water (Glass & Silverstein, 1998),
- the remediation of oil polluted sediments in the harbour of Amsterdam (Hakstege & van Geldermalsen, 1998),
- the toxic effects of surfactants and their products on methanogenesis in an aerobic microbial community (Shcherbakova *et al.*, 1999),
- the biodegradability of test substances as a closed bottle test – OECD 301D and 306 (Stagg *et al.*, 1996),
- the 'ultimate' anaerobic biodegradability of organic compounds – International Organisation for Standardisation. Draft International Standard 11734 (Stagg *et al.*, 1996),
- the biodegradation of nonylphenol ether carboxylates, octylphenol ether carboxylates, and nonylphenol (Staples *et al.*, 1999),
- the biodegradation of ^{14}C-DIPN (Yoshida & Kojima, 1978b), and
- the effect of nutrient and surfactant enhancement on the biodegradation of chlorinated hydrocarbons (Zhang *et al.*, 1998).

The activated sludge used in this study was collected from the Portswood Wastewater Treatment Works, Southampton, on the day of use. A vessel was used to take samples of the mixed liquor from the first stage activated sludge tank, where the sludge has a retention time of six hours. This plant has a dry weather flow of 23 779 m^3/day. Of a total population equivalent contribution of 70 593, 9 % of the total can be attributed to hospitals, light industry and the University of Southampton (Gabor, 2000, *pers. comm.*). It has been shown that

the transfer of the microorganisms from the wastewater treatment works to the laboratory does not result in a change in the population dynamics or the metabolic functions of the AS (Kaiser *et al.*, 1998).

The AS was transported back to the Department of Civil and Environmental Engineering in a rinsed, sealed plastic container, and was used within three hours of collection. Prior to sub-sampling from the collection container, the effluent was homogenised to ensure that both flocs and liquid effluent were used. Rather than using the same AS for all the experiments in the study, it was decided to use fresh sludge for each separate batch of experiments. Although this does introduce an uncontrolled variable, the alternative of storage by freezing the AS and defrosting it when required has been shown to introduce additional problems. Freeze/thawing not only results in deactivation of the microbes, but also causes the death of some species (Chu *et al.*, 1999). It is not known precisely what fraction of the population freezing would permanently deactivate and it is likely that this deactivation would not be reproducible.

3.2 The use of bioreactors in biodegradation research

The term bioreactor refers to a 'fermentor or other apparatus used for bioconversions' (Singleton & Sainsbury, 1987). The advantage of a bioreactor is that conditions within can be controlled to optimise degradation (Atlas, 1977, 1995), as microorganisms and their enzymes can only function within certain physiological limits (Liu & Suflita, 1993). When conditions are optimised by modifying the environmental parameters, such as temperature, aeration and nutrient levels, the process is known as biostimulation (Margesin & Schinner, 1998).

Set-ups for biodegradation experiments in bioreactors vary greatly, with each author using the available equipment, and conditions suited to the study being undertaken. Table 3.1 provides a comparison of some biodegradation studies that have been carried out which have comparable aims to this study.

As can be noted from the table, a wide variety of conditions are given in the literature. However, the basic essentials of a microbial source, water, oxygen,

carbon source, phosphorous and utilisable nitrogen source (Atlas, 1977; Rosenberg *et al.*, 1992) are provided in all the aerobic studies. Although studies given as examples have used a batch reactor, some experiments are also carried out in sequence batch reactors.

In this study stirred aerobic batch reactors were chosen as they are simple to operate, and problems and interferences from reactor hydraulics are avoided (Ubay Çokgör *et al.*, 1998).

3.2.1 Nutrients

Organic chemical biodegradation is often limited by the availability of nutrients such as nitrogen and phosphorus (Atlas, 1977, 1981, 1995; Bartha & Atlas, 1987; Boehnke *et al.*, 1998; Dart & Stretton, 1980; Floodgate, 1984; Leahy & Colwell, 1990; Minas & Gunkel, 1995; Pritchard & Costa, 1991; Santas *et al.*,1999; Shiaris, 1989). The addition of organic nutrients and mineral salts stimulates the growth of the appropriate microbial community thus increasing the natural rate of biodegradation (Al-Hadhrami *et al.* 1997, Atlas, 1977, 1981, 1991, 1995; Hoff, 1993; Jobson *et al.*, 1974; Lindstrom *et al.*, 1991; Margesin & Schinner, 1998; Rycroft *et al.*, 1994; Zhang *et al.*, 1998). Biostimulation in the form of nutrient enhancement is a widely documented and utilised tool to maximise biodegradation. It is carried out both in the laboratory and field. Nutrient mixtures used depend greatly on the target microbes, and therefore vary extensively.

Study Aim	*Bioremediation of anionic surfactants and fuel oil*	*Phenanthrene degradation*	*Biotransformation of nonylphenol ethoxylate surfactants*	*Biodegradation of chlorinated hydrocarbons*	*Biodegradation of DIPN*	*Effects of zinc and copper on activated sludge*	*Efficacy testing of oil spill bioremediation products*
Duration of experiment	35 days	31 days	180 days	Not specified	21 days	70 days	25 days
Bioreactor type	500 mL conical flask	2 L Erlenmeyer flasks	5L flasks	250 mL Erlenmeyer flasks	5 L flask	2L flasks	500 mL respirometer flasks
Sampling times	0, 3, 7, 14, 21, 28 and 35 days	0, 3, 7, 11, 15, 22, 28 and 31 days	Daily to day 8, then more sporadically	Not specified	0, 7, 14 and 21 days	Intensively to 30 d then sporadically	Start, middle and end of O_2 consumption
Nutrients added?	Some only	Basal salt medium	Yes	Basal salt medium	Yes	Bacterial growth medium	Basal salt medium
Aeration?	No	No	No	No	Yes, onto top	Yes, air sparger	No
Controlled light source?	No	No	12 hour dark-light cycle	Yes, continuous		Yes, contiuous	No
Microbial source	Soil bacteria	Stream bacteria	Activated sludge	Activated sludge	Activated sludge	Activated sludge	Sea water
Mixing supplied	Continuous	2 x daily	Continuous	Continuous	Continuous	Continuous by aeration	Continuous
Temperature	10 °C	25 ± 1 °C	21 ± 2 °C	25 ± 2 °C	25 ± 3 °C	25 °C	20 °C
Open/Closed system?	Open	Open	Open	Open	Closed for CO_2 collection	Open	Closed for CO_2 collection
Additional notes	Water losses corrected for by regular topping up	pH maintained 6.8-7.0		Sequential increase in carbon source conc.		Magnetic stirrer used, conditions optimised	
Reference	Margesin & Schinner (1998)	Romero *et al.* (1998)	Di Corcia *et al.* (1998b)	Zhang *et al.* (1998)	Yoshida & Kojima (1978b)	Cabrero *et al.* (1998)	Venosa *et al.* (1993)

Table 3.1 Examples of experimental set-ups for aerobic biodegradation experiments

A standard nutrient medium composition was chosen for addition to bioreactors at the start of experiments containing similar components to those used in other studies (Table 3.2).

Table 3.2 Nutrient mix compositions (mg/L of distilled water) in aerobic biodegradation studies using activated sludge

Compound added	Abd-Allah & Srorr (1998)	Zhang *et al.* (1998)	Shcherbakova *et al.* (1999)	This study
$CaCl_2$	-	0.022	10	7
KCl	250	-	-	-
$MgCl_2$	-	0.19	-	-
$MnCl_2$	-	0.252	-	-
NaCl	-	-	-	250
NH_4Cl	3000	-	280	125
K_2HPO_4	1000	5800	250	-
KH_2PO_4	-	4500	-	1500
Na_2HPO_4	-	-	-	3000
$FeSO_4$	2	-	-	-
$MgSO_4$	250	-	10	123
$(NH_4)_2SO_4$	-	2000	-	-
Na_2MoO_4	-	0.041	-	-

It is clear from the comparison that the essential nutrients required are ammonium, chloride, potassium, phosphate, sulphate, magnesium, and to a lesser extent calcium and sodium, with concentrations varying markedly. The nutrient mix used in this study meets all of these requirements. It is important to note however that when certain metals reach toxic threshold concentrations they may start to negatively affect biodegradation in the bioreactor (Bragg *et al.*, 1994). This is illustrated in a study into the effects of zinc and copper on activated sludge bacteria growth kinetics (Cabrero *et al.*, 1998). The authors found that single or combined concentrations of these metals up to 10 mg/L in the influent waters resulted in up to a 5 % reduction in treatment efficiency in a continuous activated sludge plant. However, copper and zinc in low concentrations act as micronutrients and are essential for the composition of special enzymes in the microbes.

3.2.2 Temperature

Biodegradation is strongly temperature dependent (Melin *et al.*, 1998). Temperature influences the metabolic activity of oxygenase enzymes involved in biodegradation (Atlas, 1981; Bartha & Atlas, 1987; Dart & Stretton, 1980; Espeche *et al.*, 1994; Hawkes, 1983; Minas & Gunkel, 1995). ZoBell (1969, as cited by Atlas, 1981) found that hydrocarbon degradation was increased by over an order of magnitude on going from 5 to 25 °C. In water, a rise of 10 °C, within the range of 0 - 40 °C, will result in a reaction rate increase of between two and three fold for most enzymes (Dart & Stretton, 1980). In addition a study by Melin *et al.* (1998) showed that a 10 °C increase in temperature resulted in over seven times faster degradation rates for 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol.

At low temperatures there is often a long lag phase observed prior to the onset of biodegradation due to the continuing presence of toxic volatile components in the organic mixture (Bartha & Atlas, 1987). At high temperatures inhibition of metabolic activity can also occur. Senez (1962, as cited in Mayhew & Stephenson, 1997) found that a progressive increase in the respiratory activity of the bacteria *Aerobacter aerogenes* was shown up to a temperature of 42 °C, beyond which inhibition was apparent.

For the purpose of this study bioreactors were placed on the bench top under a window. All bioreactors used were of the same design and colour to ensure equal light and thermal balance. Initial experiments were carried out to determine biodegradative losses without temperature control. If adequate an biodegradation rate could be achieved under these conditions then process costs would be minimised. A further set of experiments to optimise temperature settings was planned as a second phase. A type K thermocouple was used to measure temperature.

3.2.3 Oxygen

3.2.3.1 The necessity of oxygen in aerobic biodegradation

The presence of molecular oxygen also plays a crucial role in rapid mineralisation (Shiaris, 1989), as the first step in bacterial degradation of most

organics is the introduction of molecular oxygen to form products that can then be more readily used in central metabolic processes (Atlas, 1977, 1981, 1995; Bartha & Atlas, 1987; Cerniglia, 1984; Gibson, 1980; Leahy & Colwell, 1990; Rosenberg *et al.*, 1992). Anaerobic degradation of organics proceeds only at negligible rates in nature (Atlas, 1981; Bartha & Atlas, 1987; Hambrick III *et al.*, 1980; Leahy & Colwell, 1990). For hydrocarbon degradation, about 3.5 g of oxygen are required for the complete biodegradation of 1 g of oil (Bartha & Atlas, 1987). Jamison and co-workers (1975, as cited in Atlas, 1981) even showed that nutrient addition without aeration in the remediation of gasoline contaminated groundwater failed to stimulate biodegradation, hence indicating that oxygen is one of the primary limiting factors. In the activated sludge process dissolved oxygen levels are never allowed to fall below 0.5 mg O_2 L^{-1} for carbonaceous oxidation. These figures take into account the gradient through the floc to the site of respiration in the individual cells (Hawkes, 1983).

In this work a compressed air line ending in a porous diffuser stone in each bioreactor facilitated continuous aeration. An adequate volumetric airflow rate, at a flow rate of 17±2 L/min was supplied in order to maintain saturated oxygen conditions in the medium. Not only was this used to introduce a steady stream of air, but it also facilitated turbulent mixing within the bioreactor. This combination aeration and mixing system has also been used effectively in several other studies (Al-Ghusain *et al.*, 1994; Cabrero *et al.*, 1998; Tichý *et al.*, 1998). Stirring not only ensures a homogenous solution but the higher shear forces act to reduce the particle size optimising nutrient supplies (Hellenbroich *et al.*, 1999) and a combination of aeration and mixing has also been shown to increase mineralisation rates (Jee *et al.*, 1998).

3.2.3.2 Oxygen consumption to characterise biodegradation

Several methods used to quantify biodegradation focus on the use of oxygen in the process. Venosa and co-workers (1993) used a respirometer to test the effectiveness of oil spill bioremediation products. This technique provides large quantities of automatically collected data in a short time period that can be used in the laboratory to infer biokinetic rates. The respirometer measures oxygen consumption, a measure of momentary metabolic activity (Sekoulov & Heinrich,

1981), which although it gives an indication of microbial activity, cannot be used as an alternative to individual component analysis (Stagg *et al.*, 1996; Vaishnav & Korthals, 1991). In addition, this measure does not provide information about the distribution of microbial activity or the viability in the population (Griebe *et al.*, 1997).

Oxygen is required at each stage of the biodegradation of organic matter (Figure 3.1).

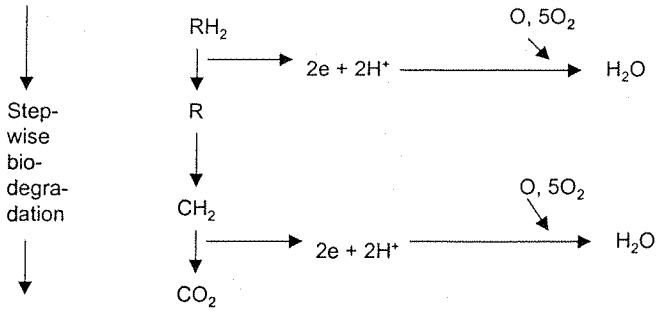


Figure 3.1 Example of stepwise aerobic biodegradation of organic matter (Sekoulov & Heinrich, 1981)

The measure of oxygen consumption is the biological oxygen demand (BOD). This is the amount of dissolved oxygen required for the microbial oxidation of biodegradable matter in an aquatic environment. The BOD_5 test measures the oxygen consumption (mg) per litre of liquid in 5 days at 20 °C (Singleton & Sainsbury, 1987).

Another measure of oxygen consumption is the chemical oxygen demand (COD). The COD test is used to measure the oxygen equivalent of the oxidisable organic matter. Oxidation extent is measured by using a strong chemical oxidant, such as potassium dichromate, in an acid medium at elevated temperatures. The Cr^{3+} produced is then quantified by titration with ferrous ammonium sulphate. The principle reaction is given by the following equation:

$$\text{Organic matter } (C_aH_bO_c) + Cr_2O_7^{-2} + H^+ \longrightarrow 2Cr^{+3} + CO_2 + H_2O$$

It has been found that the readily biodegradable, the slowly biodegradable and the inert COD fractions in relation to the total COD are a useful characterisation method for industrial water (Ubay Çokgör *et al.*, 1998).

In this study, several biological demand (BOD) experiments were carried out. They were set up according to a standardised protocol, with the sample volume used depending on the anticipated oxygen consumption level to ensure that system saturation did not occur. The airtight equipment consists of a brown bottle and an 'oxygen head', the latter of which contains the pressure sensors required for analysis.

The chosen sample volume was placed in the brown bottle. The bottle was sealed and incubated for 5 days at 20 °C prior to downloading the data from the oxygen head. The system works on the basis that carbon dioxide produced by the metabolism of organics is removed from the headspace by the sodium hydroxide pellets, resulting in a drop in pressure. The pressure decrease causes pressure compensation by the pressure sensor, delivering oxygen into the vessel that is proportional to the carbon dioxide produced initially, hence giving a measure of the oxygen consumption.

3.2.4 pH

Bacteria are also sensitive to pH conditions in their environment. Most heterotrophic bacteria and fungi favour a pH near neutrality (Espeche *et al.*, 1994; Leahy & Colwell, 1990; Ubay Çokgör *et al.*, 1998), although this can vary markedly depending on their natural conditions.

In several experiments the pH within the bioreactor was controlled (Al-Ghusain *et al.*, 1994; Cabrero *et al.*, 1998; Glass & Silverstein, 1998). pH variations of a solution give direct information about changes in hydrogen ion concentration. Tagger and co-workers (1983) found that during the oxidation of crude oil there was a decrease in pH by 3.5 units. This change was attributed to the oxidation products, such as benzoic or naphthoic acid, being more polar and soluble in water than the starting material. Measuring the pH is also frequently used to monitor nitrogen removal processes (Al-Ghusain *et al., 1994)*, where both

nitrification and denitrification will affect the pH by the respective destruction and formation of alkaline species. When the mixture in the bioreactor is subjected to aeration, equilibrium pH is usually maintained as determined by the alkalinity and carbon dioxide content of the activated sludge sample (Gernaey *et al.*, 1998).

In this work pH was measured continuously using probes attached to an automatic data logger. Sealed gel Russell combination pH electrodes (type CE711) were used for measurements taken every 10 seconds and recorded as a 15-minute average. Due to limited availability of probes, measurements were only carried out in four of the bioreactors at any one time. In addition to pH measurements, the data logger was also used to measure oxygen flow rate and temperature in one bioreactor. The data logger used was a Dataelectronics Datataker DT505.

3.3 Initial set up of the bioreactor

The initial degradation experiments were carried out using the apparatus shown in Figure 3.2. Data gathering of aeration rate, pH, and temperature occurred using the data logger.

Initial experiments included the continuous monitoring of oxygen saturation, however problems were soon noticed with the oxygen probe. The wire matrix on the bottom of the sensor became a site of microbial growth, resulting in clogging of the membrane. An alternative oxygen probe, free from these problems could have been acquired but only at great expense. Therefore, oxygen saturation is assumed due to continuous aeration of the system by use of a compressed air supply, hence no further oxygen saturation measurements were taken.

Preliminary experiments were carried out using a simple air pump. NPE analysis of several subsamples by electrospray ionisation mass spectrometry (ESI-MS) showed variable data, indicating heterogeneity within the bioreactor. This was overcome by using a stream of air bubbles from a compressed air supply to the bottom of the vessels facilitating mixing and aeration.

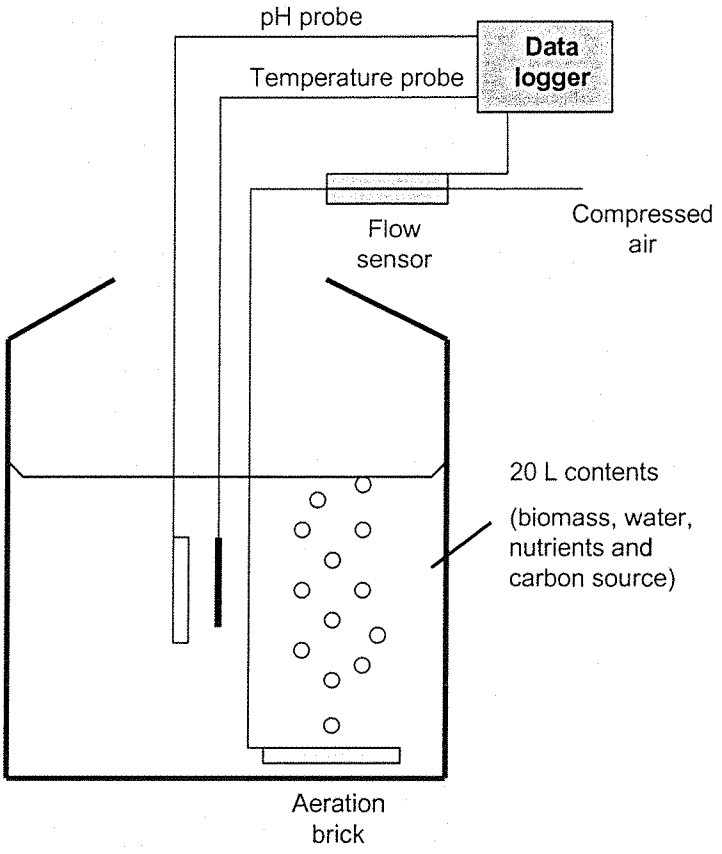


Figure 3.2 Schematic diagram of bioreactor set up, including sensors used for measurement of key parameters

3.3.1 Stage 1 experiments

These were conducted in a set of four bioreactors. The use of bioreactors in parallel allowed direct comparisons to be made. The bioreactors had a volume of 30 L with a liquid content of 20 L, composed of activated sludge, Gold Star™, nutrient medium (details given in Table 3.2) and distilled water.

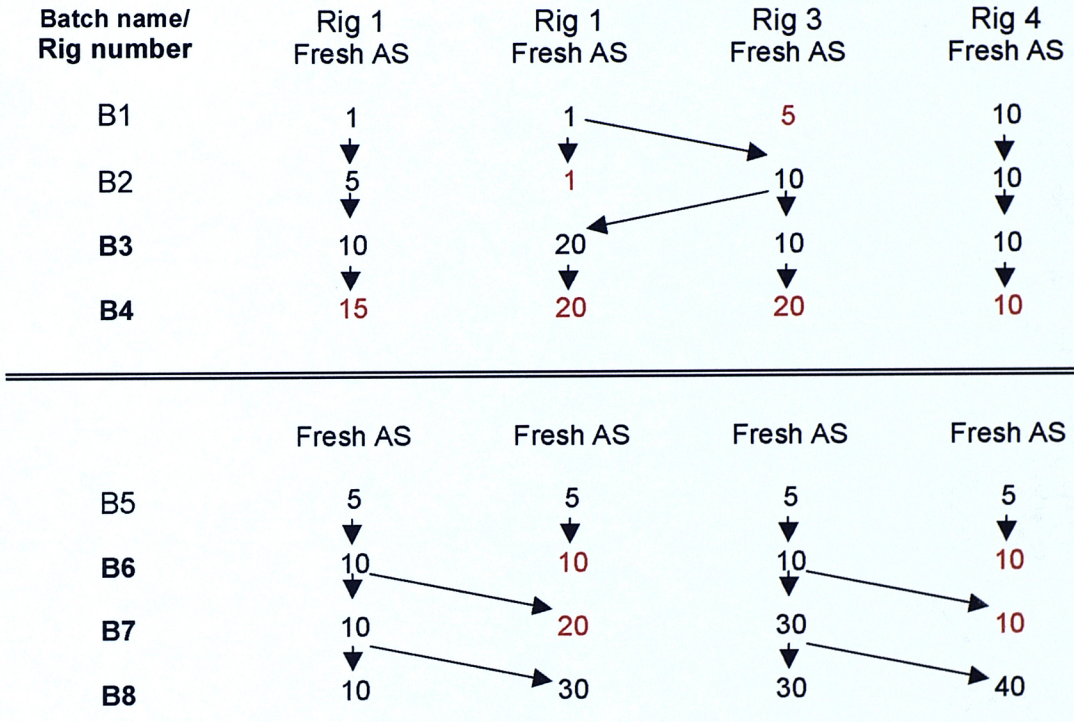
Work under stage 1 aimed to investigate and answer the following research questions;

1. Is degradation of the main analytes occurring?
2. Are all analytes degrading i.e. is Gold Star™ biodegradable in its entirety?
3. Are variations in pH an indication of microbial activity, can pH be used as an indicator of the stage of degradation?

4. What is the maximum Gold Star™ concentration that the microbial population can survive and thrive in? Is this maximum concentration affected by the adaptation pattern of the biomass?

To answer these questions biodegradation runs were set up as shown below (Figure 3.3).

Gold Star™ Concentration (%)



Red print indicates the scraping of biomass after the batch run
Arrows (⟶) indicate biomass used for each batch i.e. flow of biomass

Figure 3.3 Diagram to show the Gold Star™ concentrations and biomass adaptation patterns used in Stage 1 experiments

Stage 1 (targeting questions 1, 2 and 4) involved the analysis of samples by GC-MS. Samples were stored, but due to problems with the analytical equipment, these could not be analysed. As a result the initial intention of a feedback system between each batch, where the results from one batch aided decision making on the next step to be undertaken, did not include GC-MS data.

3.3.2 Stage 1 outcomes

pH was continuously monitored and trends were evident. Despite the presence of activated sludge, the aeration and subsequent nitrification may have resulted in a build-up of nitric acid, acting to overcome the limited buffering capacity. The overall decrease in pH with time may therefore be, in part, due to the gradual acidification of the rig contents by nitrification. However, there are clearly additional factors influencing the pH of the system, as the changes seen are not simply a gradual decrease in pH. It therefore seems most probable that despite nitrification playing a role, pH changes are additionally being influenced by biological changes in the bioreactor, including biodegradation of the carbon source by the microorganisms.

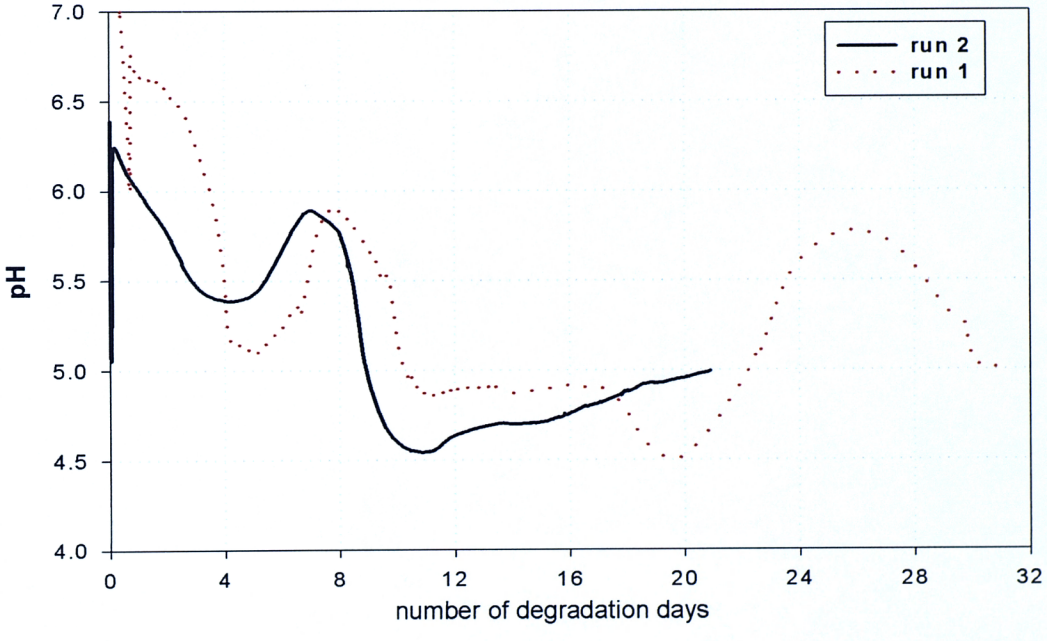


Figure 3.4 pH data for batches 1 and 2 with 10% Gold Star ™ loading

Data from the first two batches in rig 4 (10% Gold Star™ loading) are shown in Figure 3.4. A leftward shift of the pH profile in the second batch probably indicates a more rapid onset of degradation, with a subsequent constant rate of pH change. With the use of the same biomass for batches 1 and 2, it is highly likely that adaptation of the biomass between batches has resulted in a reduced lag time prior to the commencement of degradation. Thereafter changes in pH

are most likely due to the onset of biodegradation and hydrogen ion concentration changes.

Further analysis of the pH data indicated that the same increases and decreases in pH were occurring in the majority of the biodegradation experiments, albeit on different timescales. Initial data for rigs 1 and 3 contained evidence of electrical spiking in the power supply to the pH meter. This was shown by the simultaneous development of large anomalous data peaks in both pH profiles. A high impedence (>10^{14} ohms) JFET amplifier was built to isolate the pH electrodes, enabling them to be connected directly to the data logger. This change eliminated the need for the pH meters previously used, and so excluded further electrical noise in the pH data.

Initial biological oxygen demand data from stage 1 indicated that the biomass was still showing signs of growth, even at 40% Gold Star™ loading. Growth was most significant after the biomass had been adapted at several different liquid scintillant-loading levels prior to being subjected to this elevated concentration. Maximum biological oxygen demand was observed when the biomass had been adapted with more gradual increases in carbon source concentration as in rig 2 batch 8 (5, 10, 10 and 30 % GS) as opposed to faster increases as in rig 3 batch 8 (5, 10, 30 and 30 % GS). This suggests that prior exposure to equally elevated concentrations does not provide the optimum adaptation pathway, but rather that longevity of exposure at non-toxic levels is of greater importance.

Acclimation in the form of adaptation is an increase in the specific oxidising potential of the microbial community (Leahy & Colwell, 1990). Adaptation is essential in cases where microbes are used to biodegrade high concentrations of pollutants that would otherwise be toxic. It must be noted however that even if the microorganisms are adapted to cope with these elevated concentrations, sufficient supplies of oxygen and nutrients are still essential to prevent the accumulation of the pollutant to toxic levels (Ahn *et al.*, 1998). As was shown by this study, gradual adaptation of the biomass makes it more able to deal with these environmental extremes (Atlas, 1981; Bartha & Atlas, 1987; Glass & Silverstein, 1998). Details of adaptational responses are given in Section 3.1.1.

The pre-acclimation of a microbial population is used extensively in biodegradation studies to reduce the lag time prior to onset of degradative losses. Investigations using this technique include: Buitrón *et al.* (1998), Lapham *et al.* (1999), Melin *et al.* (1998), Sözen *et al.* (1998), Ubay Çokgör *et al.* (1998) and Zhang *et al.* (1998).

Due to the lack of GC-MS data, no conclusions could be made about whether degradation of the main analytes and/or Gold Star™ as a whole was occurring. However, biomass growth was still evident at very elevated concentrations and pH profiles (as shown in Figure 3.4) did appear to be reflecting changes in the bioreactors. In addition, these experiments indicated that additional data collection was necessary to further investigate the process. For a more complete data set there was therefore a necessity for several bioreactors to be run in parallel.

3.4 Biodegradation experiment developments

3.4.1 Stage 2 experiments (4 L biodegradation reactors – batch A)

Smaller bioreactors with a total volume of 6 L and liquid holding capacity of 4 L were used for stage 2. As for stage 1 experiments, this volume was composed of activated sludge, a carbon source e.g. liquid scintillation cocktail, distilled water and nutrient medium. All bioreactors had a compressed air supply to ensure aeration and mixing. Due to limited monitoring equipment availability, only certain key bioreactors were continuously monitored for pH, aeration rate and temperature. In addition, manual pH measurements are made in all bioreactors on a discrete basis.

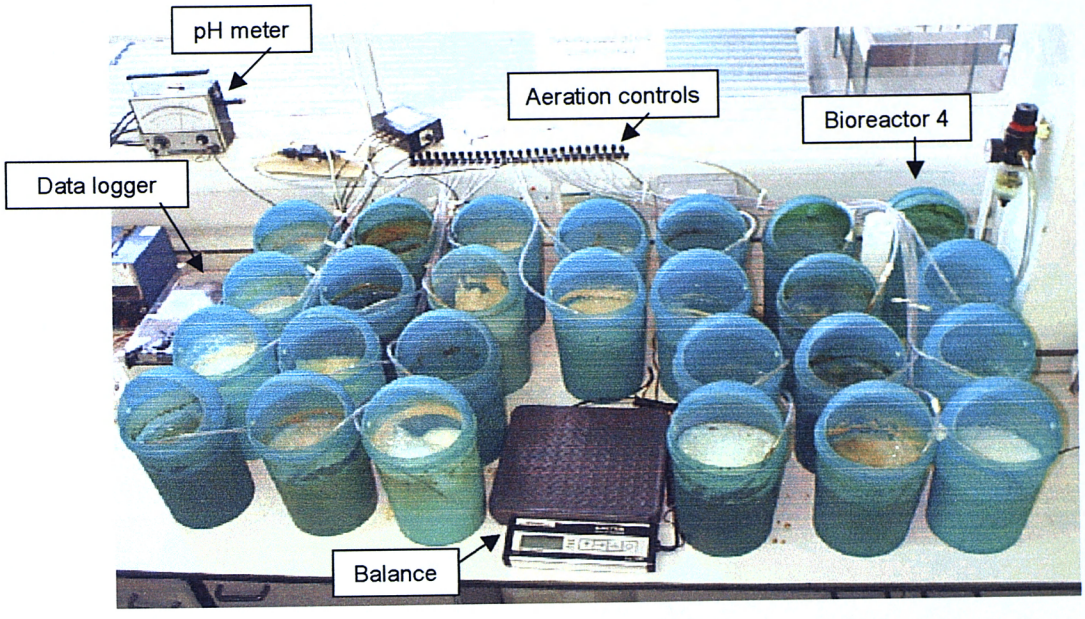
Stage 2 (batch A) research questions included the following:

1. Are the key analytes (PPO, BEE, DIPN, Bis-MSB, BAB, and NPEs) biodegradable?
2. Is EcoSafe™ (ES) biodegradable in its entirety or just certain of its components?
3. Is Gold Star™ (GS) biodegradable in its entirety or just certain of its components?
4. Is any lack of biodegradation as a result of low levels of viable microbes?

5. Is the biodegradation reproducible?
6. Do abiotic losses contribute to concentration decreases?
7. Is there a standard pH profile for the biodegradation of each component?
8. Can the pH profiles of the individual components be combined to form that of GS and ES?

Experiments were set up (Figure 3.5), and run over 100 days, with different carbon source contents and parameters being measured in each bioreactor (schematic). The photograph was taken on day 100 of the batch run. On looking at the biomass growth and content colour it is evident that final bioreactor contents varied markedly. Variations in content colour are as a result of differential interactions between the biomass and carbon source.

To answer questions 1, 2, 3, 5 and 6, analyte concentration data was required which was obtained by GC-MS, details of which are given in Chapter 5. Question 4 required data about microbial enumerations. This is covered extensively in Chapter 4. General results suggested that BEE, PPO and BAB losses were occurring. However it was unclear what proportion of this loss was as a result of microbial metabolism and what was due to abiotic losses such as volatilisation and sorption.



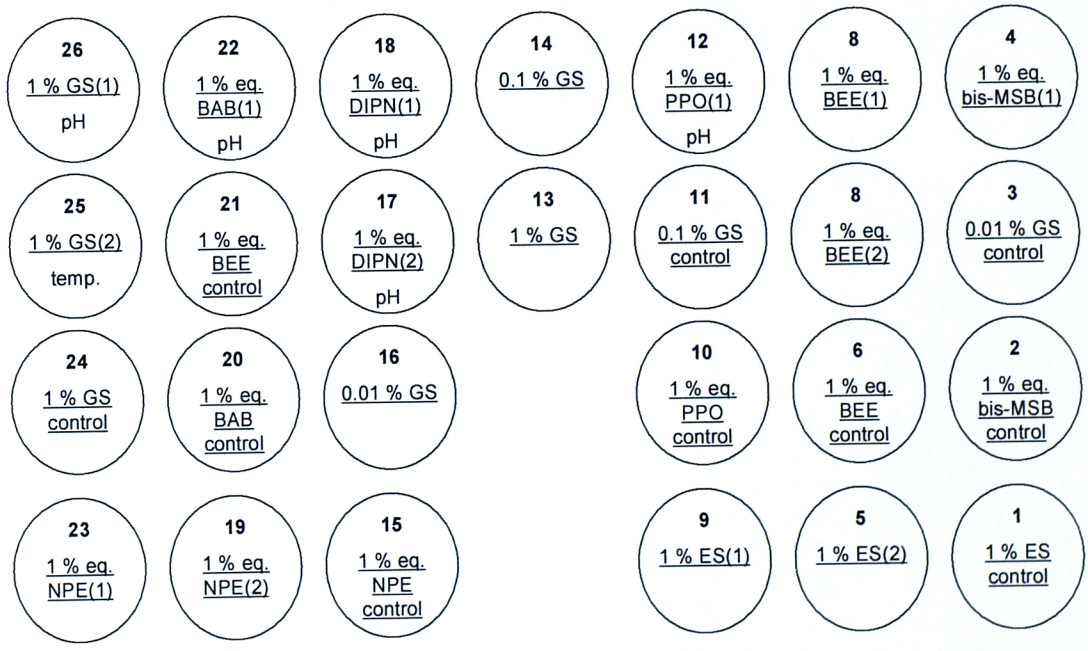


Figure 3.5 26 bioreactor set up with carbon source supplied (details underlined) and parameters (aeration rate, pH and temperature) monitored for stage 2 experiments

Control bioreactors do not contain AS and therefore will only undergo losses from photo-oxidation, volatilisation and airborne contaminating microorganisms

Key - 1% eq. = equivalent analyte concentration to that found in 1% Gold Star™ or EcoSafe™

3.4.2 Stage 3 experiments (4 L control reactors – batch B)

Abiotic controls are an integral part of biodegradation experiments. The most common method of determining losses not due to microbial respiration is carried out by exclusion of the microbial source. This arrangement was used effectively by Staples *et al.* (1999), Vaishnav & Korthals (1991) and Venosa *et al.* (1993). Due to constant conditions, it is assumed that abiotic losses are constant in all cases, and that additional changes are solely due to microbial phenomena (Tagger *et al.*, 1983).

Although copper sulphate was used in this study, other antibacterial agents have been used elsewhere. Stagg *et al.* (1996) used 100 ppm mercuric chloride to 'poison' sediment samples in order to assess losses other than those due to biodegradation. They found however that losses were not impaired by the addition of this bactericide, suggesting that the effective toxicity had been reduced as a result of sediment binding effects, making it less potent. They went on to suggest that heterotrophic plate counts at an early stage would be useful to determine the effectiveness of the antibacterial agent. Mercury at a concentration of 20 mg/L was also used by Kirby (1994) and Di Corcia *et al.* (1998b), whilst 20 mg/L of thiourea was used to inhibit microbial metabolism by Pollard and co-workers (1998).

Another method to determine abiotic losses is the addition of a similarly structured compound to the carbon source that is resistant to biodegradation. This can then be used to quantify and correct for any abiotic losses. This technique has been used to great effect for hydrocarbon biodegradation. Hopane is used as the resistant hydrocarbon (Bragg *et al.*, 1994; Venosa *et al.*, 1996), where it acts as an internal conserved standard.

The methods described above all deal with distinguishing between biotic and abiotic losses, but do not go on to quantify the different types of abiotic loss. Abiotic losses can be considered to be additive (Grady *et al.*, 1997).

Sorption onto the biomass has been shown to be an important factor in abiotic losses (Al-Lihaibi & Ghazi, 1997; Ashley & Roach, 1990; Battistoni *et al.*, 1993; Grady *et al.*, 1997; Kerr & Capone, 1988; Matsui *et al.*, 1998; Tsezos, 1986). Matsui *et al.* (1998) looked at the removal of micro-pollutants by activated sludge and found that a significant proportion of the pollutant was removed from solution by sorption. Sorption can take place with either dead or living biomass (Tsezos, 1986). It is with the significance of sorption in mind that stage 3 included an experiment to quantify this factor. Pollutant sorption site will vary depending on pollutant type. Battistoni *et al.* (1993) found that when using activated sludge the cell was the preferred sorption site for mercury, the capsular extracellular protein and cell for nickel, and the slime extracellular protein for cadmium.

Although controls without activated sludge had been included in stage 2 of this study, more detail was required regarding the abiotic loses, resulting in the setting up of a further set of experiments. This final stage of experiments also provided an opportunity to assess degradation reproducibility and to carry out a more detailed sampling programme over certain key times e.g. 0 – 10 days.

Stage 3 (batch B) questions included:

1. Are the degradation profiles found in stage 2 reproducible with data produced from experiments where AS is taken on a different day?
2. What analyte losses in GS and ES can be attributed to sorption onto the biomass?
3. What analyte losses in GS and ES can be attributed solely to biodegradation?
4. What analyte losses in GS and ES can be attributed to biodegradation by airborne contaminating microorganisms?
5. What analyte losses can be attributed to other abiotic losses?

To investigate these questions bioreactors were set up in the following format (Table 3.3):

Table 3.3 Bioreactor set up for stage 3 experiments (batch B) showing bioreactor contents and potential loss mechanisms

Bioreactor contents	*Row number*	*Biotic losses with viable biomass*	*Sorptive losses onto biomass*	*Losses due to airborne contami-nating microbes*	*Other (Photo-oxidation and volatilis-ation)*
AS + 1 % LSc	4 (back)	√	√	√	√
Autoclaved AS + 1 % LSc	3		√	√	√
Autoclaved water + 1 % LSc	2			√	√
Autoclaved water + 1 % LSc + $CuSO_4$	1 (front)				√

Copper sulphate ($CuSO_4$) was added an antibacterial agent at a concentration of 0.25 g/L
Key: LSc = liquid scintillant cocktail

A set of four bioreactors was set up with GS (left) and ES (right) and run over 28 days (Figure 3.6).

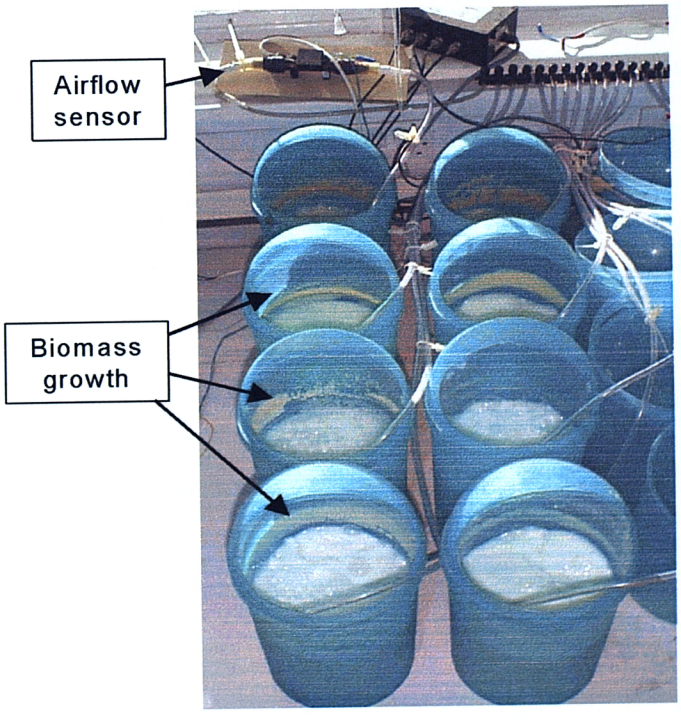


Figure 3.6 Bioreactor set up for stage 3 experiments.
Photograph taken on day 30 showing biomass growth on the sides of the containers and variable bioreactor content colour

3.4.3 Sampling procedure and sample storage

The definition of sampling is the obtaining of a sample that is representative of the bulk material, in a form and amount that is suitable for analysis. It is one of the most important steps of any analytical procedure, and errors made here negate all efforts undertaken to ensure quality during the remainder of the process (Wagner, 1995). Sampling was carried out using a 15 cm length of inert plastic tubing connected to a 10 mL syringe to obtain about 10 mL of liquid. The bioreactor subsample was put into a labelled glass liquid scintillation vial and stored immediately. All volumes sampled were recorded in order that total volume removed could be determined. This was then taken into consideration when the bioreactors were topped up on a weekly basis with milli-Q water to compensate for evaporative losses of water. This topping up was carried out 1 hour prior to sampling to allow thorough mixing. For periods of greater than a few hours prior to analysis, storage of the samples was in a freezer set at –20°C to halt all microbial action in the vial. Prior to use, sample vials were removed from the freezer and left overnight to defrost in a refrigerator. If samples were to be used within a few hours of sampling the vials were maintained at 4 °C in a refrigerator. Although this extends the possible holding time of the samples, the risk of contamination makes it less suitable for storage times of more than 24 hours (Karlsson *et al.*, 1999). Following use, the remainder of the sample was returned to the freezer in case re-analysis was required in the future.

The temperature used for the storage of samples in this study (–20 °C) is considered sufficient to halt microbial action, and was also used by Mulder *et al.* (1998), Peña-Méndez *et al.* (1999) and Sekela *et al.* (1999). The storage of samples at 4 °C for several hours is also used by Di Corcia *et al.* (1998b). The study carried out by Karlsson and co-workers (1999) into the influence of filtration, preservation and storing on organic acids in natural waters concluded that filtration using polycarbonate membranes is crucial for low level analysis. For durations up to 24 hours refrigerated storage is only effective following sterile filtration and freezing at –20 °C is required for longer time periods.

Detailed results from Stage 2 and 3 are discussed in the Chapters 4 and 5. Unlike for the continuous pH monitoring in stage 1, manual pH measurements did not show any clear trends. However the data show that the changes occurring in the duplicate bioreactors were reproducible.

Chapter 4 – Assessment of microbial changes in activated sludge for biodegradation studies

In order to investigate the biodegradation of liquid scintillant cocktails two main aspects were investigated i.e. changes in microbial biomass and compound concentration. This chapter tackles the first consideration, providing an overview of the microbiological aspects of the project. Variations in analyte concentration are discussed in Chapter 5.

4.1 The use of microbiology in biodegradation studies

Microbiology is used extensively in biodegradation research to supply information regarding microbial population dynamics, to interpolate contaminant losses through biomass growth and identify species responsible for the biodegradation of pollutants.

Table 4.1 Biodegradation studies that have included microbiological aspects

Microbiological measure undertaken	*Study type*	*Reference*
Bacterial counts by plating	• Effects of amendments on the microbial utilisation of oil	• Jobson *et al.* (1974)
	• Biodegradation of nonylphenol ethoxylates by river microbial consortia	• Maki *et al.* (1996)
	• Biodegradation of nonylphenol ethoxylates	• Mann & Boddy (2000)
	• Monitoring biodegradation by soil biological activities	• Margesin *et al.* (2000)
	• Effect of microbial seeding of crude oil in seawater	• Tagger *et al.* (1983)
	• Effects of petroleum on estuarine and marine microorganisms	• Walker & Colwell (1975)
	• Biodegradation rates of petroleum components	• Walker *et al.*(1976)
Population kinetics	• Degradation of synthetic organic compounds	• Magbanua *et al.* (1998)
Oxygen uptake	• Comparing respirometry with catalase activity of biomass in activated sludge	• Guwy *et al.* (1998)
	• Monitoring bioremediation by soil biological activities	• Margesin *et al.* (2000)
ATP uptake	• Microbial respiration in activated sludge	• Griebe *et al.* (1997)

It is evident that microbiological methods are used for many different applications (Table 4.1), but despite the wide variety of possible analyses, only some of which are included, the relatively standard and simple method of bacterial counts by agar plating is still most extensively used.

The analysis of microbial growth for biodegradation studies involves the culturing of microbes to produce colonies that are subsequently quantified. Bacterial growth occurs by a budding, binary fission or sexual mode. The predominant mechanism is binary fission, where cell division results in the original cell becoming two new organisms (Tchobanoglous & Burton, 1991).

Growth rate of a colony differs during the various stages of growth. At the beginning there is usually a lag phase, or phase of adjustment, whilst the single cell acclimatises to the conditions and a second layer of cells is gradually formed. Following this the colony increases exponentially in both diameter and height (exponential phase), with biomass increase occurring at a rate similar to the maximum specific growth rates of the colony. This maximum growth rate of a microorganism is a function of generation time and ability to process food (Tchobanoglous & Burton, 1991). It is this phase that is primarily used for growth rate determination (Sorokin, 1973).

The onset of the limiting phase, or declining phase, occurs when nutrient diffusion to the centre of the colony is limited, and/or there is a build up of toxic products. This instigates a decrease in growth rate with further growth being limited to the peripheral zones of the colony, and a resulting linear increase in the diameter of the colony. This linear radial growth has been observed for colonies of several bacteria, yeast and fungal species, but is not apparent for swarming bacteria (Salvesen & Vadstein, 2000). The transition to this third stage of colony growth is dependent on the species involved and the environmental conditions, but has been shown to occur for certain microbes at a colony diameter of approximately 0.2 - 0.3 mm (Plomley, 1959, as cited by Salvesen & Vadstein, 2000).

The stationary phase is where a stable concentration of microbial mass per unit volume is maintained, usually there is an equilibrium between the growth of new cells and the death of old ones. The final stage of the microbial culture is the death phase. Overall this growth cycle can be symbolised by a sigmoidal growth curve (Sorokin, 1973) (Figure 4.1). It is important to note that this simplified model concerns a single population of microorganisms only. When several different species are present within a colony under optimised conditions, overlapping growth cycles and variable microorganism predominance with time will greatly complicate the situation.

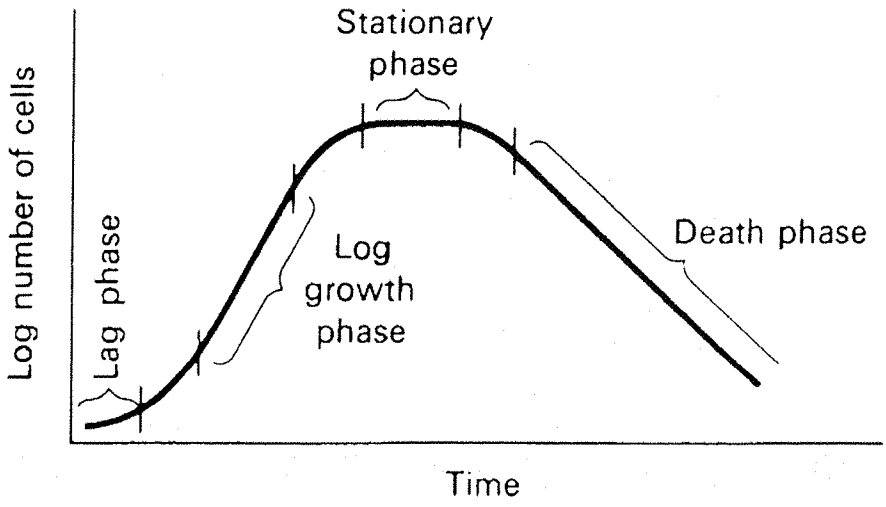


Figure 4.1 Growth phases in batch cultures of microbial organisms (Source: Tchobanoglous & Burton, 1991)

Taking the growth dynamics of microbial cultures into account, dry weight, cell number and volume of cells per unit volume of medium can be used as measures of cell growth (Guwy *et al.*, 1998). The latter thus lends itself to a measurement in turbidity changes of a bacterial suspension by means of optical density measurements (Sorokin, 1973), assuming there is no interference from particles other than microbial cells (Guwy *et al.*, 1998). In this study agar plating for biomass quantification and optical density measurements for biomass quantification and growth dynamics were used.

4.2 The use of agar and replica plating

As mentioned in Section 4.1, agar plating is used extensively for microbial enumeration. Buitrón and co-workers (1998) used agar plating to isolate bacteria responsible for the biodegradation of chlorophenols. They therefore plated activated sludge biomass onto agar containing only chlorophenol as a carbon source. Guerin & Jones (1989) and Romero *et al.* (1998) also used this procedure for phenanthrene-degrading bacteria. In addition to this role, agar plating is often used as a means of estimating bacterial concentration and innoculum condition prior to setting up biodegradation experiments. This enumeration approach was utilised for *Pseudomonas aeruginosa* prior to respiratory experiments on the biodegradation of alkanes by Al-Hadhrami *et al.* (1997), and Margesin & Schinner (1998) in the study of bioremediation of wastewater contaminated with anionic surfactants and fuel oil. Kirby (1994) used agar plating to monitor biomass changes during oil dispersant biodegradation where sampling and plating out of bioreactor biomass was carried out periodically.

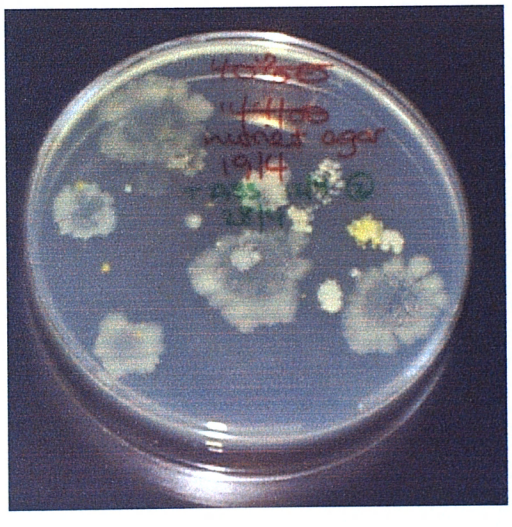


Figure 4.2 Activated sludge biomass cultivated on nutrient agar, with evidence of different colonies

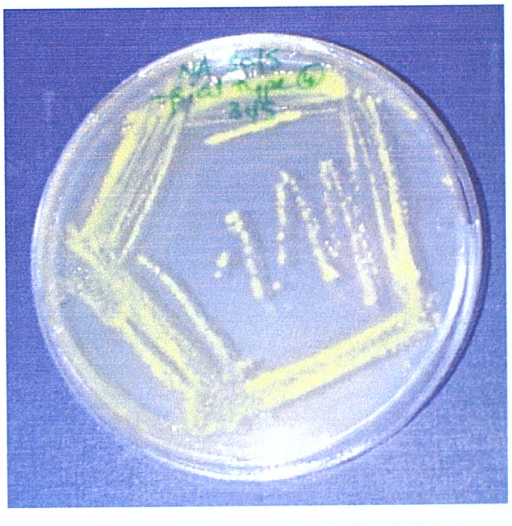


Figure 4.3 Agar plate showing microbial growth following wire loop spreading

Agar plating involves a serial sample dilution under sterile conditions followed by the spreading of a small subsample onto solidified gel consisting of agar and essential growth nutrients. Petri dishes containing the solidified gel and sample

are left to develop over a specified period of time. The time chosen is sufficient for colony formation, where each colony is assumed to be the progeny of a single cell (Figure. 4.2). Colonies are subsequently counted and related to original sample microbe concentrations in terms of colony forming units per unit volume (n), according to the following formula:

$$n = a \times 10^{b+1}$$

where; a = average number of colony forming units per plate (for countable plates containing 30 - 300 colonies), and

b = number of dilutions (undiluted: b = 0, 1 in 10 dilution, b = 1 etc).

The sterile transfer of the cultivated colonies from original agar plates onto others can be carried out by picking with a wire coil or replica plating. Wire coil picking involves dipping a sterile wire coil into the colony of choice and then wiping this lightly in a Z pattern onto a fresh agar plate to spread out the individual cells (Figure 4.3). This technique is often used to purify a bacterial colony for use as pure culture or identification purposes. Replica plating involves the use of a sterile sheet of velvet that is pushed on to the surface of an agar plate with colonies growing on it. The velvet is then gently pressed on to a fresh agar plate to transfer all the colonies. This technique is primarily used to maintain a culture of microbes or to determine whether or not specific cultures can be grown on agar containing different growth substrates. Unfortunately transfer by replica plating often results in smudging of the colonies, hence new colonies grow into diffuse patches rather than discrete points.

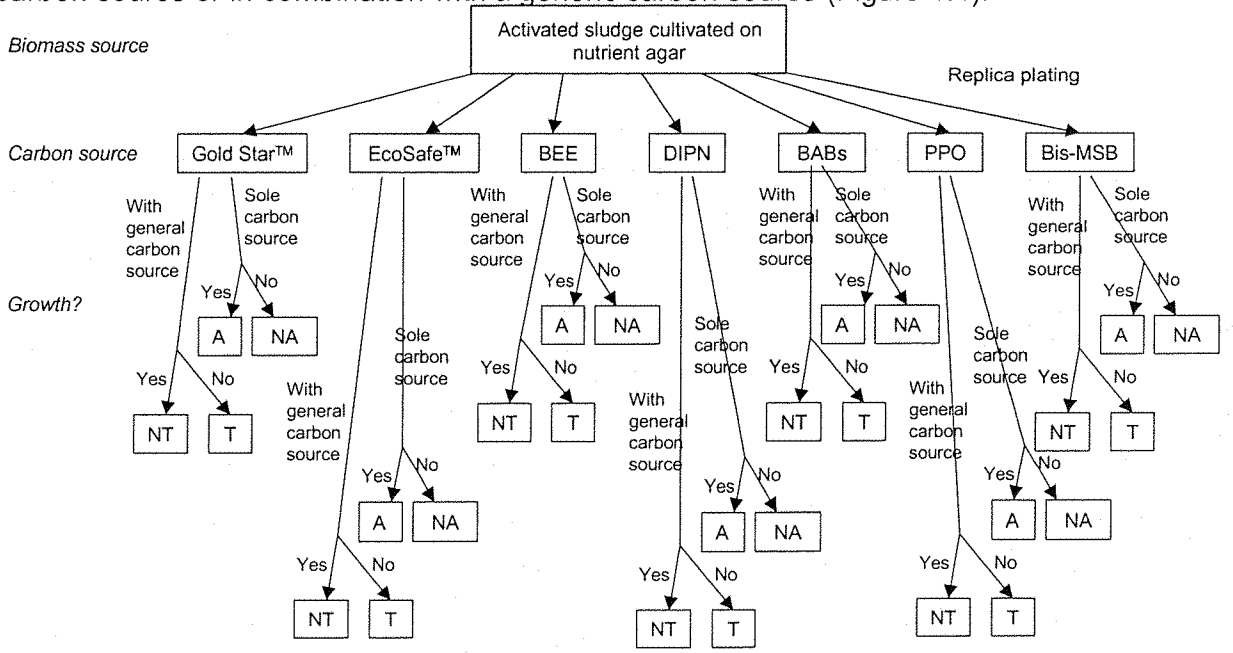
Agar plating is seen as an indirect counting method (Dugenest *et al.*, 1999), particularly when used with an unselective agar such as nutrient agar, containing general nutrients and carbon sources such as peptone and yeast extract (Tylor, 1996). Agar plating was used in this study to identify and quantify the microbial biomass thriving in the bioreactors and to establish a link between changes in biomass and analyte concentration.

4.2.1 Research questions

Agar plating aimed to investigate and answer the following more specific research questions:

i. can all individual analytes and the liquid scintillant cocktails themselves be used as carbon sources?
ii. which of the compounds is the best carbon source, thus producing maximum biomass growth?
iii. do specific microbial types adapt to specific carbon sources?
iv. what is the maximum carbon source concentration for biomass growth?
v. do specific analytes inhibit growth?

In order to answer these questions, several different aspects had to be investigated. Activated sludge (AS) biomass was initially grown on nutrient agar plates. The colonies were then replica plated onto agar plates containing individual compounds or liquid scintillant cocktails (LS cocktails) as a sole carbon source or in combination with a generic carbon source (Figure 4.4).



Key: A growth evident, therefore available for metabolism
NA no growth, therefore not available for metabolism
T inhibits growth, therefore toxic
NT doesn't inhibit growth, therefore not toxic

Figure 4.4 Microbiological tests to be conducted and possible outcomes Note – the activated sludge was cultivated on agar plates containing a general carbon source. Following this the colonies were transferred to the specific carbon sources shown by replica plating.

4.2.2 Agar and replica plating protocols

Plating protocols vary depending on the aims of the work and the specific microbial populations involved (Table 4.2). Cultivation parameters of 20 °C for 7 days in the dark were chosen for both the agar and replica plating in this study.

Table 4.2 Incubation temperatures and durations

Reference	*Incubation temperature (°C)*	*Incubation time*
Cicek *et al.* (1998) *	37	24 hours
Dugenest *et al.* (1999)	30	5 days
Yuan *et al.* (2000)	30	21 days
Romero *et al.* (1998)	25 ± 1	30 days
Spain *et al.* (1980)	25	5 days
Jobson *et al.* (1974)	21	6 days
Kirby (1994)	20	48-96 hours
Tagger *et al.* (1983)	20	3 weeks
Minas & Gunkel (1995) **	18	3 weeks
Stagg *et al.* (1996)	12	7 days
Margesin & Schinner (1998)	10	2 weeks

Key: * - using Standard Method 907 of the APHA Standard Methods for the Examination of Water and Wastewater
** - carried out in the dark

The techniques used for this study have been adapted from Tylor (1996). Details of protocols used are given in Appendix 3. Despite slight detail variations, the procedures used are comparable to those used in other studies including Romero *et al.* (1998), Mann & Boddy (2000), and the standard method for the counting of viable bacteria from the Dutch Government Publishing Office (1980).

4.2.3 Method development and evidence for biodegradation of liquid scintillant cocktails

Summarised results of all plating experiments are given (Table 4.3). Method development and significance of these results are discussed in the subsequent sections.

Table 4.3 Agar plating results

Carbon source used	*Microbial biomass source*	*Growth occurring after 7 days?*	*Relevant section for discussion*
Nutrient agar	Distilled water	Yes	4.2.3.1
Nutrient agar	Milli-Q™ water	No	4.2.3.1
Nutrient agar	Nothing	No	4.2.3.1
Nutrient agar	Glass rod smear	No	4.2.3.1
None	AS	No	4.2.3.1
Autoclaved AS	AS	No	4.2.3.2
Nutrient agar	AS	Yes	4.2.3.2
0.1 – 30 % GS	Cultivated AS microbes *	No	4.2.3.3
0.1 – 10 % ES	Cultivated AS microbes *	No	4.2.3.3
Nutrient agar	1 % GS bioreactor biomass	Yes	4.2.3.3
1 % GS	1 % GS bioreactor biomass	No	4.2.3.3
0.1 – 0.5 % GS	1 % GS bioreactor biomass	No	4.2.3.3
0.1 – 0.5 % ES	1 % GS bioreactor biomass	No	4.2.3.3
Nutrient agar and 1 % GS	1 % GS bioreactor biomass	Yes	4.2.3.3
Nutrient agar and 1 % eq. PPO	Cultivated AS microbes *	Yes	4.2.3.3
Nutrient agar and 1 % eq. bis-MSB	Cultivated AS microbes *	Yes	4.2.3.3
1 % eq. BEE	Cultivated AS microbes *	Yes	4.2.3.3
1 % eq. DIPN	Cultivated AS microbes *	No	4.2.3.3

Key: eq. concentration equivalent to that present in a 1 % LS cocktail solution
AS Activated sludge
GS Gold Star™
ES EcoSafe™
PPO diphenyl oxazole
Bis-MSB bis-methylstyryl benzene
BEE butoxyethoxy ethanol
DIPN di-isopropyl naphthalene
* replica plated from activated sludge cultivated on nutrient agar

4.2.3.1 Agar plating control plates

Control plates were used for each batch of agar-plating to assess environmental and procedural sources of contamination. Control plates included incubation of nutrient agar plates with distilled water, Milli-Q™ water and no plated samples. Figure 4.5, 4.6, 4.7 show the absence of growth on the plates with no additions following pouring (plate preparation control), UV irradiated Milli-Q™ water and

distilled water respectively. In addition, the sterilised glass rod was smeared over the surface of a nutrient agar plate without any sample having been introduced. As for Milli-Q™ water and the 'no sample' this showed no biomass growth after 7 days (Figure 4.8).

Test result: sterile plate preparation occurred, but the distilled water did contain some culturable microorganisms

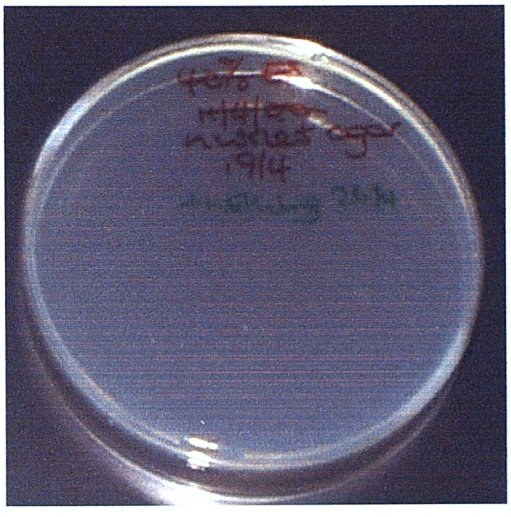


Figure 4.5 Nutrient agar with no sample – no growth

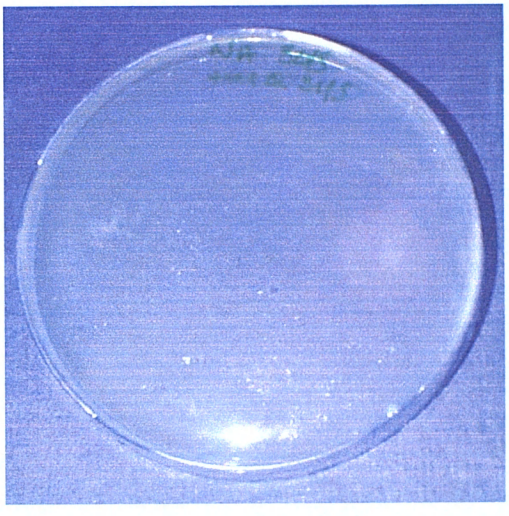


Figure 4.6 Nutrient agar with UV-irradiated Milli-Q™ water – no growth

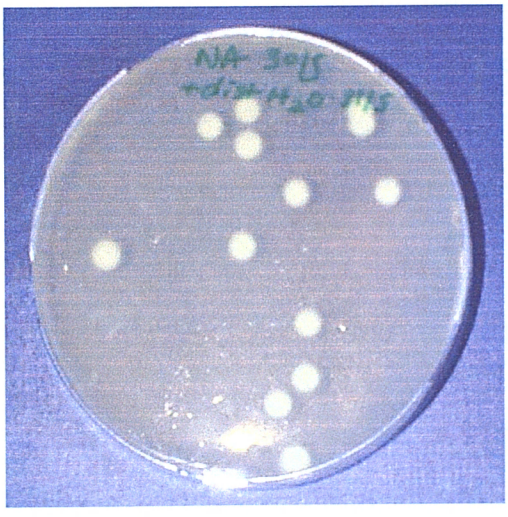


Figure 4.7 Nutrient agar with distilled water – distinct colonies present

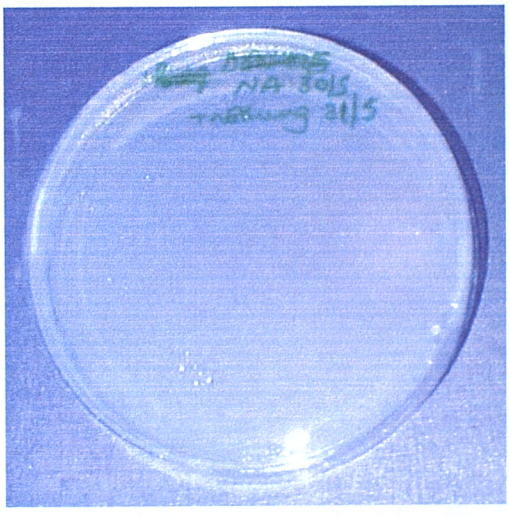


Figure 4.8 Nutrient agar smeared with glass rod only – no growth

Plates were prepared with a carbon-free agar mixture and essential nutrients to test a theory by Floodgate (1984). He noted that some microbial species are able to grow on the low concentrations of organic nutrients found as impurities in the media components used to make up the agar mix. No growth of AS biomass cultivated on these plates was evident.

Test result: there is insufficient carbon present for growth in the pure agar and nutrient mixture

4.2.3.2 The use of activated sludge as a microbial source

No growth of AS biomass on agar plates prepared with autoclaved activated sludge was observed. However, growth was evident when AS was plated onto nutrient agar, with an average and standard deviation of 2.8×10^5 and 1.5×10^4 colony forming units per mL (CFU/mL) respectively, each of these colonies having developed from a single microbial cell.

Test result: activated sludge can be cultivated and does contain viable microorganisms. It does not contain sufficient essential growth nutrients to maintain a microbial population, whereas the nutrient agar does

On inspecting the colonies formed it was evident that fourteen types had developed. Classification was by a single characteristic shape or a combination of features (Figure 4.2). Yuan *et al.* (2000) also used colony morphology to distinguish between culture strains. As used by Romero *et al.* (1998), individual strains were transferred onto fresh nutrient agar plates using the wire loop method for further purification and growth.

Test result: fourteen different microbial species present in activated sludge when cultivated on nutrient agar

4.2.3.3. Activated sludge biomass growth on liquid scintillant cocktails and individual analytes

Following development of the AS colonies on nutrient agar at a dilution factor of 1 in 1000, replica plating was used to transfer them onto agar plates containing Gold Star™ (GS) (1-30 %) and EcoSafe™ (ES) (1-10 %) as the sole carbon sources. ES concentrations of greater than 10 % prevented the setting of the agar LS cocktail mixture and therefore could not be used. No discernible colony

development on any of the plates after two weeks suggests that AS biomass which utilise the general carbon source in nutrient agar was not capable of utilising ES or GS at these concentrations.

Test result: activated sludge cannot use Gold Star™ or EcoSafe™ as a sole carbon source

A sample of the contents from a bioreactor containing 1 % GS was diluted and wire-loop plated onto nutrient agar to assess viability of the biomass with GS as the sole carbon source, and 1 % GS agar to assess utilisation of this carbon source. Growth was only evident on the nutrient agar plates (average = 5.2 x 10^5 CFU/mL, standard deviation = 1.0 x 10^5 CFU/mL) (Figure 4.9). When classification of the cultivated colonies was carried out, it was evident that fewer types were present than those found for fresh AS (eight vs. fourteen types in fresh AS), with a predominant presence of specific strains.

Test result: natural selection of the biomass occurs when specific carbon sources are present for growth

The lack of growth on 0.1 to 0.5 % GS (Figure 4.10) and ES agar containing plates suggests that LS cocktails present in the agar mixture are either unavailable as a carbon source or are having a toxic effect on microbes even at these low concentrations. To investigate the possible toxicity of GS and ES, agar plates were prepared with both the general carbon source present in nutrient agar and an additional 1 % GS carbon source. GS bioreactor biomass growth on these plates was compared with that on simple nutrient agar. Both plates showed substantial growth, suggesting that the presence of GS does not have a toxic effect on biomass at this concentration but rather that it is unavailable. Further experiments using AS colonies replica plated on to nutrient agar plates with 1 % LS cocktail concentration equivalent of diphenyl oxazole (PPO) and bis-methylstyryl benzene (bis-MSB) showed substantial growth.

Test result: EcoSafe™, GoldStar™, diphenyl oxazole and bis-methylstyryl benzene are all non-toxic to the biomass, and therefore do not inhibit growth

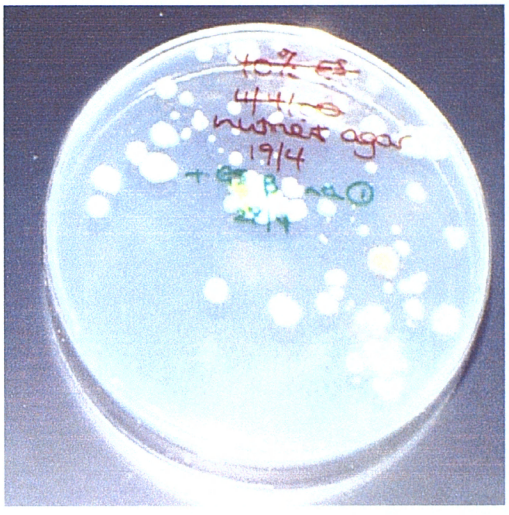


Figure 4.9 Nutrient agar with Gold Star™ bioreactor biomass – growth evident

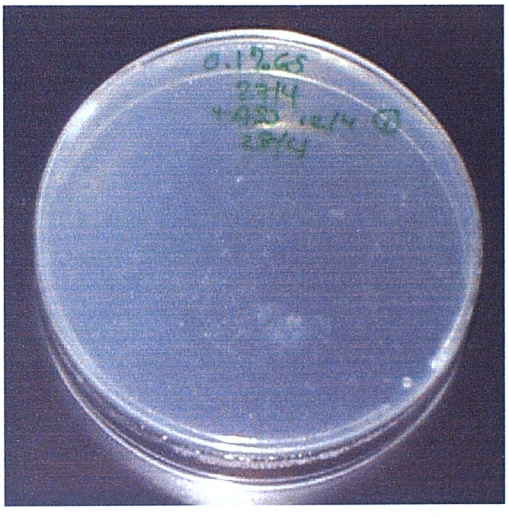
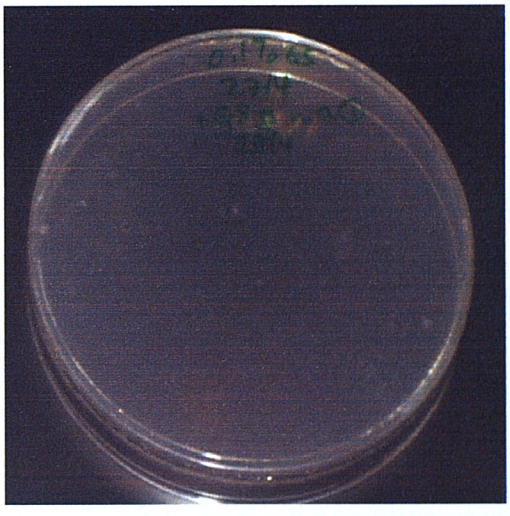


Figure 4.10 (a) 0.1 % GS agar with AS biomass – no growth



(b) 0.1 % GS agar with GS bioreactor biomass – no growth

A final toxicity experiment was carried out where nutrient agar plates with ready developed AS colonies, had disks soaked in the individual analytes and LS cocktails placed on them. The disks were small pieces of sterile filter paper soaked in 1 % LS cocktail or the concentration equivalent of the individual compounds. These disks were then placed on the colony-covered agar surface and left for 7 days to assess inhibition (Figure 4.11). No growth inhibition zones were observed around any of the compound disks.

Test result: none of the individual components or the liquid scintillant cocktails as a whole is toxic to growth

No biomass growth was apparent on agar plates containing 1 % LS cocktail concentration equivalent of DIPN (Figure 4.12). This experiment was established to determine whether or not the compound was available for use by the biomass. However, growth was evident when using BEE as the carbon source (Figure 4.13).

Test result: BEE is available for growth, whereas DIPN is not. In addition, the plate preparation protocol used does not make the carbon source unavailable

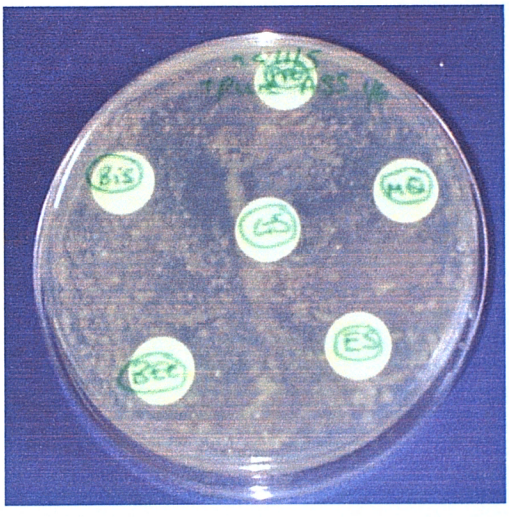


Figure 4.11 Nutrient agar with AS colonies and disks soaked in analyte solutions – no evidence of toxicity

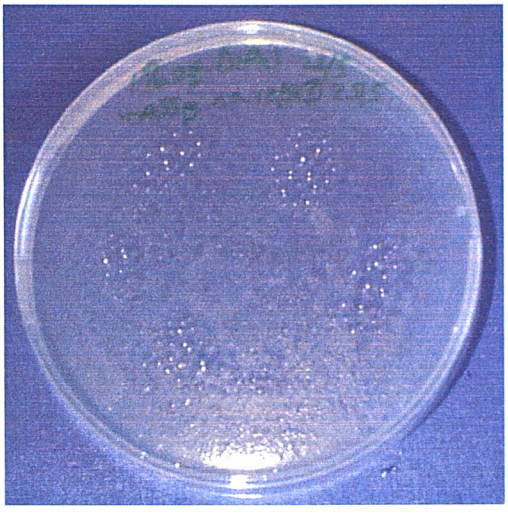


Figure 4.12 1 % eq. DIPN agar with AS biomass - no growth

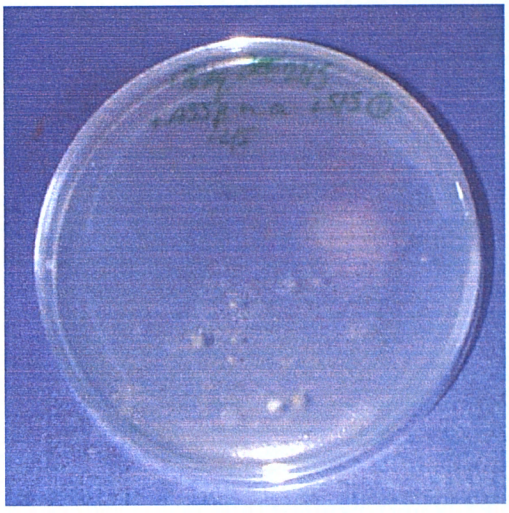


Figure 4.13 1 % eq. BEE agar with AS biomass – some growth

Key: eq. = concentration equivalent to that present in a 1 % LS cocktail solution
Note: the marks evident in Figure 4.12 are DIPN that has not thoroughly mixed into the agar jelly and therefore lays on the surface

4.2.4 Conclusions and limitations of the procedure

4.2.4.1 Conclusions

Investigations with control plates showed that:

- the nutrient agar plates were prepared aseptically,
- there was no atmospheric contamination of the plates during the cultivation period,
- the glass rod was sufficiently sterilised to prevent contamination on sample spreading,
- UV-irradiated water was free of microbes, whereas the distilled water was not, and therefore this latter source could not be used for sterile dilutions, and
- the carbon free agar mixture did not contain any trace of a suitable carbon source for AS biomass growth.

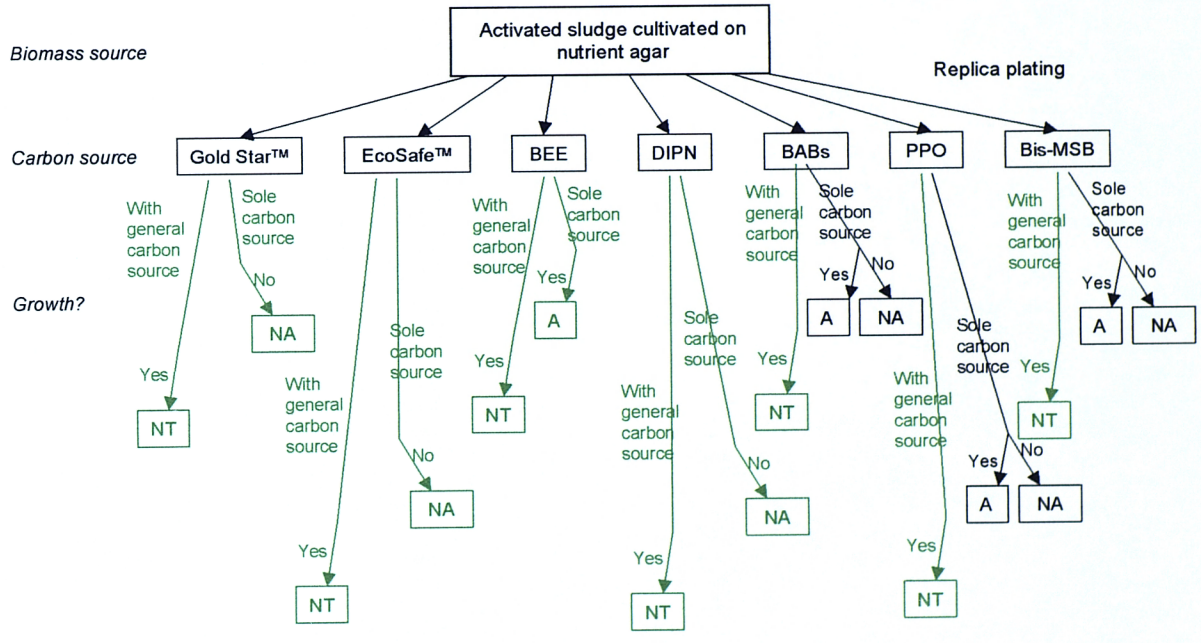
Conclusions to be drawn on the use of AS as a suitable microbial source included that:

- activated sludge itself does not contain the required energy source to maintain a viable biomass,

- activated sludge is a good source of a mixture of microbial strains, with 14 different types having been identified, and
- the wire loop and replica plating techniques are effective colony transfer mechanisms.

Experiments regarding the growth of activated sludge with the liquid scintillant cocktails and their components showed that:

- microbial strain selection had occurred in the GS bioreactors, with a reduction in the number of types found after introduction of the highly biodiverse AS biomass,
- both LS cocktails and all individual analytes were non-toxic to growth,
- BEE was metabolised and used as an energy source by the microbial community, and
- DIPN, GS and ES were unavailable for metabolic use (Figure 4.14).



Key:
- A — growth evident, therefore available for metabolism
- NA — no growth, therefore not available for metabolism
- T — inhibits growth, therefore toxic
- NT — doesn't inhibit growth, therefore not toxic
- ——— Experimental outcomes

Figure 4.14 Outcomes of agar plating experiments

4.2.4.2 Limitations of the techniques used and required developments

Quantitative analysis required to compare the use of the different carbon sources was limited by the highly variable plate count data. Various factors including multiple colony development on a single location and dilution, subsampling and counting errors could contribute to these variations. It must also be noted that this technique depends on the counting of cultured microorganism colonies. It is however the case that not all viable strains present in a sample will be culturable (Griebe *et al.*, 1997; Margesin *et al.*, 2000; Salvesen & Vadstein, 2000) and therefore enumerations do not necessarily quantify the actual biomass present in the sample. Culturable ability of a microorganism is a function of the growth media available, culture temperature and cell specifics. Nutrient agar will facilitate colony development of bacteria, fungi and actinomyces species. However, identification of genera was not performed. Of the microbial strains found in AS, it is likely that up to 40% will be viable but not culturable (Smallman, 1999, *pers. comm.)*. It is also the case that as population dynamics change a varying proportion of the total viable biomass will be culturable and thus direct comparisons cannot easily be made. The agar plating technique was therefore used primarily for a qualitative assessment where these variations were of reduced significance.

Although procedural contamination can be excluded, the data obtained by agar plating is still very variable. Therefore optical density measurements were also conducted for a less subjective assessment of biomass growth and to allow for quantitative comparisons.

4.3 The use of optical density measurements for bacterial enumerations and growth kinetics

Optical density is a measurement of turbidity, which can be correlated with cell density, and hence cell numbers. It is determined by a spectrophotometer with subsamples being aseptically taken from the bulk solution for analysis. Turbidity measurements are then correlated with optical density data and bacterial counts.

Of all indices of growth, optical density measurements are particularly suitable to monitor microbial growth. This is due to minimal and non-destructive preparation, its truly quantitative nature and the ability to take repeated measurements of the same suspension over a period of time (Sorokin, 1973). These factors result in a rapid technique that involves minimal manipulative errors.

For the purpose of this study, optical density was used to give an indication of microbial numbers (a single measurement), and for microbial population dynamics (turbidity difference with time). Microbial enumeration was used by Goudar *et al.* (1999), Larson *et al.* (1979), Mulder *et al.* (1998) and Walker *et al.* (1975), whilst the technique was utilised by Boonchan *et al.* (1998), Cabrero *et al.* (1998) and Zhang *et al.* (1998) for information regarding population dynamics .

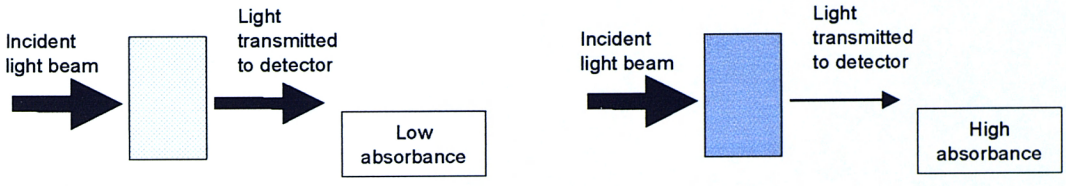
4.3.1 Objectives

To corroborate the data acquired by the agar plating, this investigation had several of the same objectives. However, in order to utilise the extended capabilities of the technique several additional objectives were included:

i. can AS biomass utilise the LS cocktails and/or the individual compounds as an energy source?
ii. which carbon source results in maximum biomass growth?
iii. do any of the analytes act to inhibit or enhance growth when combined with the general carbon source?

4.3.2 The spectrophotometer

The spectrophotometer works on the principle of detecting the remaining light from an incident light beam of a specific wavelength and intensity, which has passed through a sample. Absorbance is the fraction of the incident light that does not reach the detector and has therefore either been absorbed by the sample or deflected away from the incident beam.



The wavelength used depends on the sample type (Table 4.4). Some particles have the ability to absorb and subsequently re-emit light, a process known as fluorescence. A wavelength must be chosen at which this effect does not occur. This study used a Cecil 3000 Series Scanning Spectrophotometer, with a wavelength of 600 nm.

Table 4.4 Examples of different wavelengths used for optical density measurements

Purpose of measurement	*Wavelength used (nm)*	*Reference*
Pseudomonas putida enumerations	600	Ahn *et al.* (1998)
Activated sludge bacterial enumeration	450	Cabrero *et al.* (1998)
Aromatic bacteria enumerations	660	Goudar *et al.* (1999)
Marine bacteria growth dynamics	610	Juwarkar & Khirsagar (1991)
Pseudomonas growth dynamics	540	Mulder *et al.* (1998)
Seawater bacteria growth dynamics	660	Salvesen & Vadstein (2000)
Soil bacteria enumerations	450	Skeen *et al.* (1994)
Oil degrading bacterial enumerations	600	Walker *et al.* (1975)
Activated sludge biomass growth dynamics	540	Zhang *et al.* (1998), (1999)

For full details of the optical density measurement protocol see Appendix 3.

4.3.3 Method development and results

All experiments were initially carried out using the mixed nutrient and carbon stock solutions for dilution and zeroing purposes. However, microbial growth may result in colour changes of the medium that would change the absorbance properties of the sample. To compensate for this effect, further experiments were carried out using broth filtrate. A fraction of the growth sample was filtered using a 0.7 µm cellulose filter. The filtrate was then used for autozeroing purposes as a background subtraction for the spectrophotometer, and to dilute growth samples for use in a calibration experiment. Ahn *et al.* (1999) and Zhang *et al.* (1998) also used filtrate in this way to compensate for background colour absorbance.

4.3.3.1 Relating absorbance measurements to microbial enumerations

In order that the optical density measurements can be used to determine changes in biomass, a correlation between absorbance and population density of a given cell suspension was established (Figure 4.15). The relationship between biomass growth and changes in compound concentration is discussed in Chapter 5.

Test result: an increase in optical density can be related to a change in biomass and growth of the population

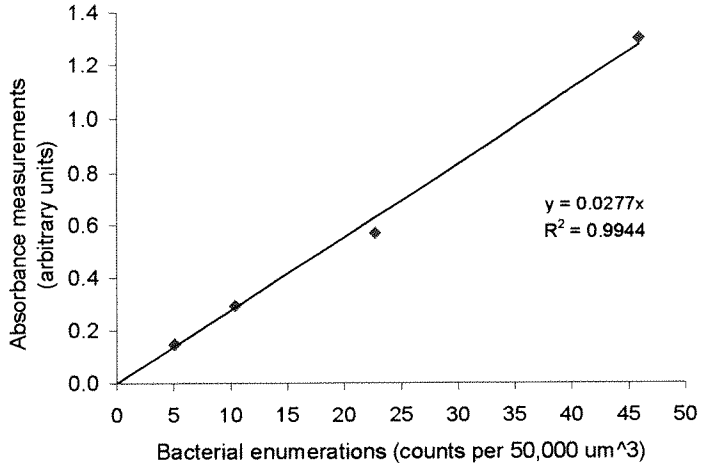


Figure 4.15 Correlation between absorption and microbial enumerations

4.3.3.2 Growth experiments to assess growth of activated sludge biomass on the compounds of interest

Experiments were conducted to determine growth dynamics in media containing the liquid scintillant cocktails or their constituents as individual carbon sources. Toxicity tests were also carried out, along with control experiments to assess contamination of the stock solutions (Table 4.5).

There was no biomass growth in any of the control experiments showing that there was no microbial contamination in any of the stock solutions. In addition these results showed that carbon stocks did not contain any nutrients and the nutrient stocks did not contain the necessary carbon source required for biomass growth.

Test result: all stock solutions were sterile and not contaminated with additional essential growth nutrients

There is no indication of differing degrees of adaptation to the various carbon sources due to the poor reproducibility of the data. Therefore the quantitative results required to determine the carbon source most readily used for growth, cannot be obtained and only general comparisons can be made.

Test result: these optical density measurements could not be used for quantitative purposes

The experiments monitored turbidity change over a period of three days. An increase in biomass and therefore turbidity will result in increased absorbance. Increased absorbance with the presence of a sole carbon source indicates that the specific source is metabolised and utilised for microbial growth, as is the case for GS, ES and BAB (Table 4.6). No substantial increase in absorbance was noticed when BEE, DIPN, PPO and bis-MSB were present as the sole carbon sources. When the general carbon source was combined with individual compound stock solutions, no discernible impact from the LS cocktails or any of the other compounds was evident.

Table 4.5 Combinations of nutrient and carbon source stocks used for the growth experiments

AS	*N*	*GC*	*BEE*	*DIPN*	*PPO*	*BAB*	*Bis-MSB*	*ES*	*GS*	*Objective number or control type*
	√									Control A & C
	√	√								Control A
	√	√	√							Control A
	√	√			√					Control A
	√	√							√	Control A
		√								Control A & B
√										Control B
√		√								Control B
√			√							Control B
√					√					Control B
√									√	Control B
√	√									Control C
√	√		√							O-1 & 2
√	√			√						O-1 & 2
√	√				√					O-1 & 2
√	√					√				O-1 & 2
√	√						√			O-1 & 2
√	√							√		O-1 & 2
√	√								√	O-1 & 2
√	√	√								O-3
√	√	√	√							O-3
√	√	√		√						O-3
√	√	√			√					O-3
√	√	√				√				O-3
√	√	√					√			O-3
√	√	√						√		O-3
√	√	√							√	O-3

Key:

Control A	to check for presence of microbes in stock solutions
Control B	to check for presence of nutrients in stock solutions
Control C	to check for presence of a carbon source in stock solutions
N	Nutrient mix stock
GC	General Carbon stock

Objectives:

O-1 to establish if AS biomass can utilise the LS cocktails and/or individual components as an energy source?

O-2 to determine which carbon source results in maximum biomass growth?

O-3 to assess if any of the analytes act to inhibit or enhance growth when combined with the general carbon source?

Test result: GS, ES and BAB are available for use as a growth nutrient by the biomass whilst BBE, DIPN, PPO and bis-MSB are not. The liquid scintillant cocktails and none of the compounds inhibit biomass growth

Table 4.6 Summarised results from the growth experiments

Carbon source	*Absorbance change with sole carbon source*	*Absorbance change when combined with general carbon source **
General carbon	Increase i.e. growth	Not applicable
Butoxyethoxy ethanol	No change i.e. no growth	No difference i.e. general growth
Di-ispopropyl naphthalene	No change i.e. no growth	No difference i.e. general growth
Branched alkyl benzenes	No change i.e. no growth	No difference i.e. general growth
Di-phenyl oxazole	No change i.e. no growth	No difference i.e. general growth
Bis-methylstyryl benzene	No change i.e. no growth	No difference i.e. general growth
Gold Star™	Increase i.e. growth	No difference i.e. general growth
EcoSafe™	Increase i.e. growth	No difference i.e. general growth

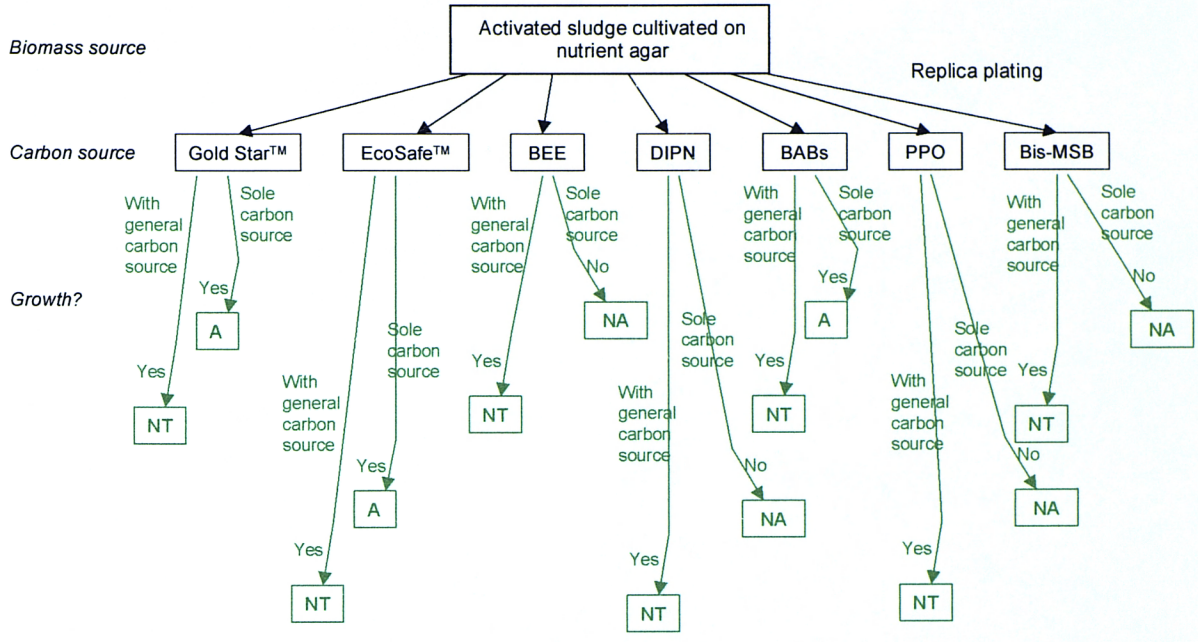
* as compared to general carbon solution as the sole carbon source

4.3.4 Conclusions and limitations

4.3.4.1 Conclusions

The following conclusions can be drawn from the optical density experiments:

- sample filtrate must be used for zeroing and dilution purposes, rather than distilled water which does not take into account any medium colour changes as a result of microbial growth,
- changes in absorbance are directly related to biomass change and can therefore be used to draw conclusions regarding population dynamics,
- GS, ES and BAB are available as a carbon source, whilst the presence of BEE, DIPN, PPO and bis-MSB does not result in substantial biomass growth, and
- no inhibitory effect on biomass growth when in combination with the general carbon source is detected with ES, GS or any of the other compounds (Figure 4.16).



Key:

	A	growth evident, therefore available for metabolism
	NA	no growth, therefore not available for metabolism
	T	inhibits growth, therefore toxic
	NT	doesn't inhibit growth, therefore not toxic
———		Experimental outcomes

Figure 4.16 Outcomes from the optical density experiments

4.3.4.2 Limitations and discussion

As for agar plating enumerations, poor data reproducibility prevented the making of quantitative comparisons as required to determine the carbon source allowing for greatest biomass growth. Relative standard deviations for triplicate growth experiments ranged from 5 to 170 %, whilst analytical reproducibility was comparatively high with relative standard deviations of 0.5 to 5.5 %. It is clear that this technique is best used as a diagnostic tool for qualitative rather than quantitative analysis.

In a discussion of the limitations of optical density measurements by Sorokin (1973), certain key factors affecting the precision of the measurements were highlighted. These include:

- non-homogenous sample,
- poor optical properties of the cuvette used,
- inappropriate wavelength used, and
- poor adjustment of the instrument.

Homogenisation of the sample prior to introduction into the cuvette was carried out in this study simply by agitating the bottle in which the sample was held. Griebe *et al.* (1997) used a glass homogeniser and Teflon piston at 300 rpm for 4 mins. This is stated as resulting in disaggregation of the biomass without disruption of the bacterial cells. It was noticed in the present study that biomass colonies readily settled to the bottom of the cuvette when placed in the spectrophotometer. Resultant errors were minimised by rapid transfer of agitated solutions, quickly followed by a measurement being taken. High analytical reproducibility of the experiments suggests that this procedure was effective in mixing the sample (typical relative standard deviations of 1 %).

To ensure optimal transparency of the sample-holding vessel, a quartz cuvette was used. Between each sample distilled water was used to rinse out the cuvette in triplicate, and excess water was shaken out of it to prevent dilution of the subsequent sample. The wavelength chosen for the study was one used by several others (Table 4.4), and was that utilised as standard in the laboratory where the experiments were conducted. Zeroing of the spectrophotometer and a substantial warm up period were used to compensate for equipment variability and to ensure comparability of the data. As for sample homogenisation, high analytical reproducibility, even when replicate samples were analysed over the period of several hours, indicate that equipment variability was minimal. A possible limitation of the optical density technique is that it is purely a measure of turbidity and not of viable cell count. It is however assumed that as turbidity change with time is used, this can be solely attributed to the living microorganisms.

4.4 Overall microbiology conclusions

Due to low experimental reproducibility, neither plate counts nor optical density measurements were appropriate for quantitative analysis. In addition, plate counts considerably underestimate the viable microbial counts (Griebe *et al.*, 1997; Margesin *et al.*, 2000), and both methods are time-consuming.

The work does show that activated sludge (AS) is capable of growth under the conditions used. It is therefore an effective and inexpensive microbial source for biodegradation studies such as this one. The large biodiversity found in AS is also shown to undergo natural selection following growth in a bioreactor containing specific carbon sources like liquid scintillant cocktails.

Regarding the microbial metabolism of the compounds in liquid scintillant cocktails, the microbial analysis was somewhat inconclusive with agar plating and optical density experiments contradicting each other on several occasions (Table 4.7). Correlation is found for most of the toxicity assessments and for all data regarding DIPN, bis-MSB and PPO. Despite triplicate optical density experiments, the outcomes for BEE are contradictory and therefore no conclusions can be drawn from this data. It will however be possible to assess losses, and therefore the metabolisation of this compound from the GC-MS data (Chapter 5). The discrepancy between the two techniques suggests that a different method of assessing microbial growth, such as the most probable number technique, may be considered for use in any further investigations.

Table 4.7 Summarised outcomes of agar plating and optical density measurements regarding compound metabolism and toxicity

Compound	*Agar Plating*	*Conclusions*	*Optical Density*	*Conclusions*
	Toxic?	*Available and metabolised?*	*Toxic?*	*Available and metabolised?*
BEE	No	Yes	No	No
DIPN	No	No	No	No
PPO	No	Not assessed	No	No
Bis-MSB	No	Not assessed	No	No
BAB	No	Not assessed	No	Yes
GS	No	No	No	Yes
ES	No	No	No	Yes

The correlation found between bacterial numbers and absorbance will also need to be further linked with compound concentration changes, as was conducted by Ahn *et al.* (1998). If a link between concentration decreases and biomass increase can be found, then the losses can, at least in part, be attributed to biodegradation and metabolism by the activated sludge biomass. If

further identification tests are conducted then the specific adapted strains to certain compounds can also be determined.

Further experiments to investigate population dynamics can be carried out to investigate relative growth rates with frequent measurement over a specific time period. Siddiqui *et al.* (1997) showed that bacterial growth following the exposure to easily biodegradable compounds reached a maximum at approximately 48 hours, whilst the bacterial growth peak was appeared after only 24 hours in a study by Kirby (1994). This suggests that measurements to assess population dynamics would need to be taken several times per day. These experiments will help to understand the dynamics of the system, including determining the lengths of each of the growth periods. When linked to compound concentration information this can then be further used with varying environmental conditions in an attempt to control the lag and exponential growth phases to maximise biodegradation effectiveness.

Chapter 5 - GC-MS studies

'Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary while the other moves in a defined direction' (Ettre, 1993).

Various chromatographic techniques exist including, gas, liquid, thin layer, ion, paper, foam and emulsion chromatography. Of this array of techniques, gas chromatography is used in this study. Gas Chromatography is a basic analytical tool in organic chemistry. It is widely used in the analysis of organic compounds that volatilise yet remain stable upon heating. The combination of gas chromatography and mass spectrometry is a widely used application to provide both qualitative and quantitative information, where the mass spectrometer acts as a detector to improve resolution, sensitivity and analytical possibilities (Castello & Gerbino, 1993; Chee *et al.*, 1996; Schröder, 1993).

This chapter provides a detailed account of the GC-MS technique used, its method development, the results obtained and a discussion of the conclusions drawn regarding the biodegradation of liquid scintillant cocktails.

5.1 Gas chromatography mass spectrometry (GC-MS) in biodegradation studies

GC-MS is well suited to the analysis of organic pollutants such as aromatic and aliphatic compounds. It has been extensively used in a number of biodegradation studies to monitor concentration changes of these compounds with time (Table 5.1).

GC-MS is utilised as it can simultaneously analyse a wide variety of analytes over a large concentration range. It is the method of choice for high-resolution separation of complex hydrocarbon mixtures with moderate to low molecular masses (Furton *et al.*, 1993), but cannot easily be used for high molecular mass, non-volatile compounds such as proteins. Non-volatile samples can however be adapted for analysis by GC-MS with derivatisation or pyrolysis prior to introduction into the gas chromatography column (Grob, 1985).

Table 5.1 The use of GC-MS in biodegradation studies

Study	*Purpose of GC-MS analysis*	*Reference*
Bioremediation of the *Exxon Valdez* oil spill	Hydrocarbon quantification with time	Bragg *et al.* (1994)
Depuration of petroleum-contaminated seashore sediments	Saturate and aromatic hydrocarbon analysis	Fusey & Oudot (1984)
Biodegradation of Phenanthrene	PAH analysis	Guerin & Jones (1989)
Alkylphenol ethoxylate surfactant biodegradation	Nonyl- and octylphenol quantification	Hawrelak *et al.* (1999)
Bacterial transformation of pyrene	Pyrene metabolite identification	Li *et al.* (1996)
Toxicity of nonylphenol ethoxylate biodegradation products	Nonylphenol ethoxylate product identification and quantification	Maki *et al.* (1998)
Biodegradation of nonylphenol polyethoxylate in river water	Identification and quantification of nonylphenol polyethoxylates	Manzano *et al.* (1998)
Bioremediation in soil	Mineral oil quantification	Margesin *et al.* (2000)
Crude oil degradation by marine bacteria	Quantification of aromatic fraction	Rontani *et al.* (1986)
Biodegradation of methylated naphthalenes	Identification and quantification of naphthalenes	van Aarssen *et al.* (1999)
Efficacy of oil spill bioremediation products	Hydrocarbon quantification	Venosa *et al.* (1993)
Petroleum-degrading potential of bacteria from water and sediment	Saturate hydrocarbon quantification	Walker *et al.* (1975a)
Microbial petroleum degradation	Analysis of the GC unresolved fraction	Walker *et al.* (1975b)
Biodegradation rates of petroleum components	Saturate concentration monitoring with time	Walker *et al.* (1976)

5.2 The GC-MS system

This is composed of an inlet, gas chromatography oven and column, and ionisation and detection systems in the form of a mass spectrometer.

5.2.1 Injectors

The function of an inlet is to receive a sample aliquot and, if it is not already in the gas phase, to vaporise it. Inlet systems must not cause sample loss or degradation and must ensure that it passes onto the chromatographic column as a single dose so as to avoid any resolution loss.

There are several types of GC injector port including:

- split/splitless,
- direct,
- on-column, and
- temperature programmable injectors.

Different injectors are used for different applications, primarily depending on the concentration and volatility of the compounds of interest.

5.2.1.1 Split/splitless injection

The split/splitless injector is the most commonly used injection system, with the introduction of high concentration samples being possible. Generally the split mode (Figure 5.1) is utilised for samples with a concentration of 100 ppm and above. Upon injection, the sample is instantaneously vaporised and a specific proportion is purged off through the open split vent, reducing the sample volume that passes onto the column. If volatilisation and mixing are not instantaneous, analytes of low volatility can be preferentially lost down the split vent and therefore do not pass on to the column for subsequent analysis. Careful quantification studies are required to characterise and compensate for these losses.

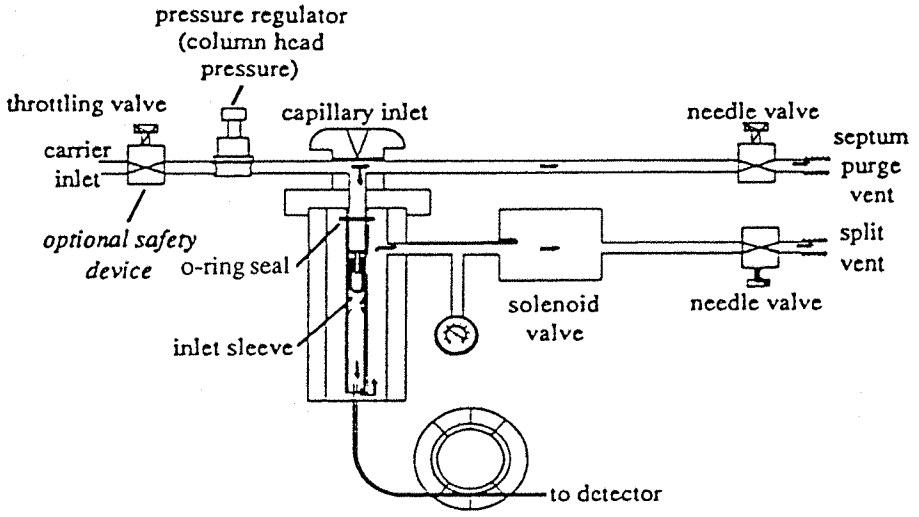


Figure 5.1 Schematic of a split injector. This shows the inlet sleeve into which the sample is injected. The solenoid valve is shown open, allowing for a known fraction of the sample to be purged out of the split vent (on the right hand side of the figure), and the column (at the bottom) conveying the sample to the detector. (Source: Restec Co., 1999)

Other than highly concentrated samples, the system is also often used for 'dirty' samples as the contamination can be trapped before passing onto the column. This is achieved by the inclusion of an inert packing material, like glass wool, in the injector liner.

In general, the splitless mode is used for the analysis of trace components in a relatively pure solvent (Figure 5.2). Following injection, flash vaporisation without any purging occurs resulting in a substantial gas volume passing onto the column. This large volume may result in limited focusing of analytes at the head of the column, broad peaks and a loss of resolution. Improved focussing of the sample is achieved by cool injection, with carrier gas flow transferring the sample into the column. A rapid increase in oven temperature then volatilises the sample and the solenoid valve is simultaneously opened to purge off any analytes remaining in the injector. A disadvantage of this system is that any undesirable compounds present in the sample will also pass onto the column, thus increasing chances of system contamination and carryover between samples.

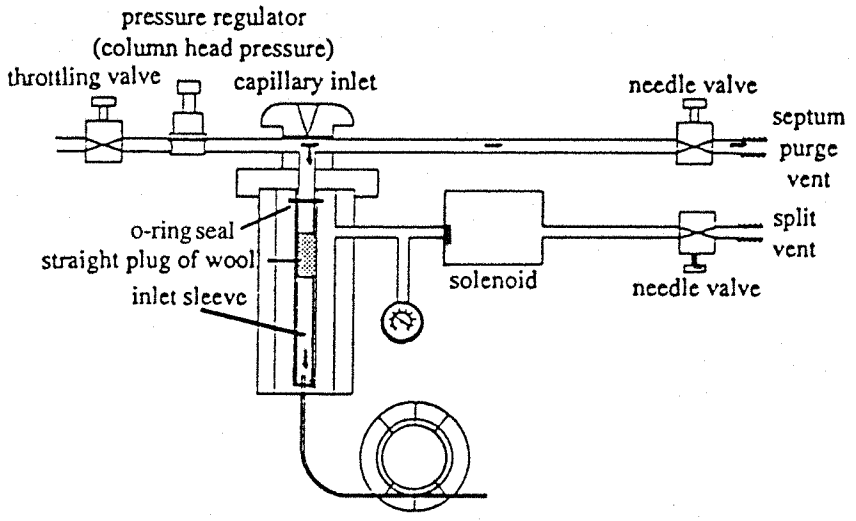


Figure 5.2 Schematic of a splitless injector. This is the same set-up as given for a split-injection system in Figure 5.1, however, in this case the solenoid valve is closed, preventing any sample loss through the split vent. (Source: Restec Co., 1999)

5.2.1.2 On-column injection

Unlike for split injection, fewer losses of volatile analytes occur if injection is made directly onto the column, where the sample vaporises ensuring an inert pathway (Figure 5.3). In order to avoid the resolution loss problems experienced with splitless injection, only very small injection volumes can be used (1 μL as opposed to 200 μL for split injectors). On inserting the needle through the heated injector, vaporisation of analytes with low flash points may occur. These analytes can then be lost either attached to the needle itself or through the septum opening as the needle is retracted from the injector (backflash). This problem can be overcome both by rapid injection and cool on-column injection, followed by a temperature increase to vaporise the sample. The cool injection method provides an inert pathway for active compounds, deposits the sample onto the head of the column where it is focused, and reduces the chances of thermal degradation and backflash. However only 'clean' and dilute samples can be injected in this manner and there is frequent mechanical damage to the column.

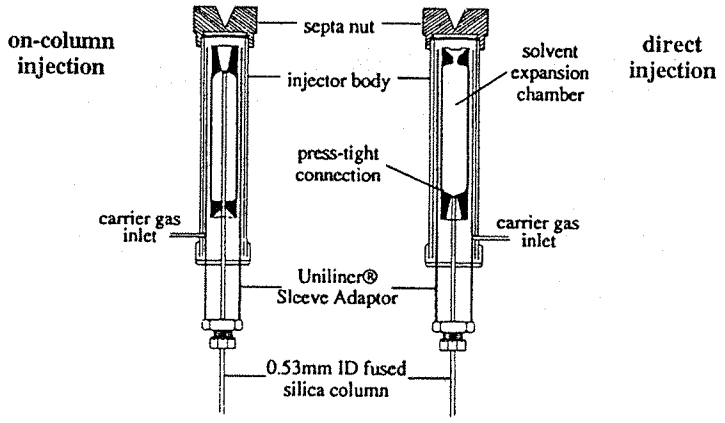


Figure 5.3 Schematic of on-column and direct injection systems (Source: Restec Co., 1999)

5.2.1.3 Direct injection

This technique involves sample vaporisation in an inlet sleeve preventing needle damage to the column phase. A further advantage of the inlet system is that it can be utilised for solvent expansion, allowing for larger volumes to be

injected and accommodated. However, the presence of an interface between injection needle and column provides a site for samples to react and be altered. Deactivated liners can be used to overcome this problem.

5.2.1.4 Temperature programmable injector

Temperature programmable injectors (Figure 5.4) combine the advantages of several of the other techniques with high concentration focusing, no selective loss of volatile fractions, contaminant removal capabilities, and the option of using split or splitless injection.

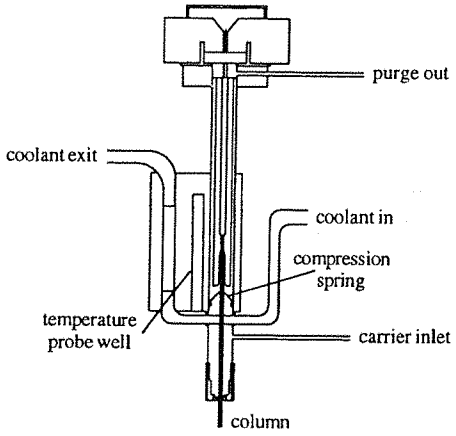


Figure 5.4 Schematic of a temperature programmable injector (Source: Restec Co., 1999)

The liquid sample is injected at a temperature below that of the lowest boiling compound, and is vaporised due to a rapid injector temperature increase. This injection technique can be effectively utilised for trace level samples and those with analytes of a wide range of boiling points. It minimises the loss and degradation of thermally labile compounds, can include injector liner packing for 'dirty' compounds, and standard syringe needle sizes can be used. It effectively operates as a 'cool' direct injection system.

5.2.2 Gas chromatography (GC) columns

The aim of the column is to separate compounds in the sample mixture before they pass into the detector. Separation is achieved by variable retention of the analytes in the stationary solid phase of the column. Partitioning of the analyte into the stationary phase is wholly dependent on the solute/phase interactions.

These include factors such as dipole-dipole interactions, van der Waals forces, hydrogen bonding, and particularly the polarity of the stationary phase. As the column polarity increases, retention times of polar compounds increase whilst those of non-polar analytes such as hydrocarbons are reduced. As compound vaporisation temperature is attained they pass into the mobile phase, the carrier gas (usually helium or hydrogen), and are carried to the detector.

In addition to stationary phase variations affecting the polarity, columns differ greatly in length, internal diameter, film thickness, and stationary phase type. They can vary between 10 - 105 m in length, with an internal diameter of 0.18 - 0.53 mm, film thickness of 0.25 – 5.0 µm, and phase composition of various polysiloxanes and polyethylene glycols. Optimisation for use with specific applications involves a fine balance of these parameters (Table 5.1), maximising peak seperation and minimising analysis times.

Table 5.1 Effect of changing GC column characteristics

Parameter to increase	*Peak seperation efficiency of early eluting compounds*	*Speed of analysis*
Length	Increase for both early and late eluters	decrease
Internal diameter	Increase	increase
Film thickness	Increase	decrease

5.2.3 The mass spectrometer (MS)

As the GC oven temperature increases, each of the analytes reaches the detector in turn where they are detected as a pulse of electrons, which are registered as a peak. All mass analysers have the following characteristics in common:

- determination of the mass of an ion, generated by an ionisation source prior to the analyte reaching the detector,
- determination of a mass-to-charge ratio (m/z), allowing for identification and quantification of individual compounds,
- measurement of gas-phase ions,
- operation at very low pressures, to ensure a free gas-phase ion path to the analyser (Willoughby *et al.*, 1998).

In this study a ThermoFinnigan Trace MS 2000^{TM} was used. Mass spectrometers are composed of the same basic units, namely a source, analyser and detector. There are many different types of these individual units. The role of the source is ion production, whilst ion separation occurs in the analyser and ion detection in the detector.

An electron ionisation source (Figure 5.5) was used. This procedure uses electron bombardment of the sample gas molecules. A tungsten or rhenium filament is heated by an electric current to a temperature at which electrons are emitted, generally with an energy of 70 eV (Grob, 1985). The electrons are attracted to an anode across the gas molecule stream emitted from the GC column, collisions with sample molecules occur causing molecular ion formation by fragmentation. The ions are then attracted out of the source to an extracting lens, passing through a focussing and accelerating lens *en route* to the analyser, the quadrupoles.

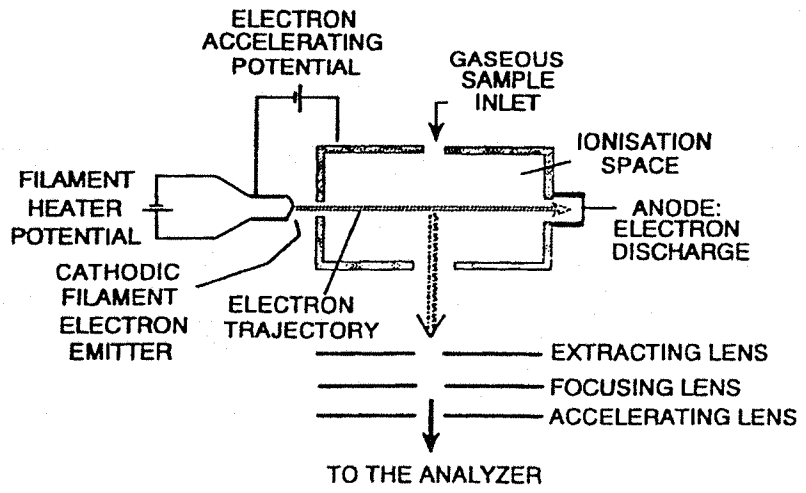


Figure 5.5 Schematic of an electron ionisation source (Source: de Hoffmann *et al.*, 1996). The diagram shows the sample inlet where gaseous molecules from the GC column are received. The electrons passing between the cathode and anode ionise these molecules. The ions are focussed by the various lenses and pass on to the analyser.

Quadrupoles (quads) consist of 4 parallel rods (poles) equally spaced around a central axis. Low energy ions from the ionisation source, via the focussing lenses, are introduced along the pole axis (Figure 5.6). Precisely controlled

voltages are applied to opposing pole pairs creating a 'mass filter', with specific positive and negative voltages creating an upper and lower limit within which there is an area of mutual stability (the bandpass region) for ions with a specific mass-to-charge ratio (m/z). It is solely these ions which are able to pass along the rod axis and on to the detector, whilst ions outside the bandpass region collide into the rods, halting their path to the detector. Ramping of the rod voltages results in a complete range of masses passing on to the high voltage detector with time. Although quads are capable of detecting an upper mass of 4000 Da, they are best used for applications of up to 1000 Da (Willoughby *et al.*, 1998).

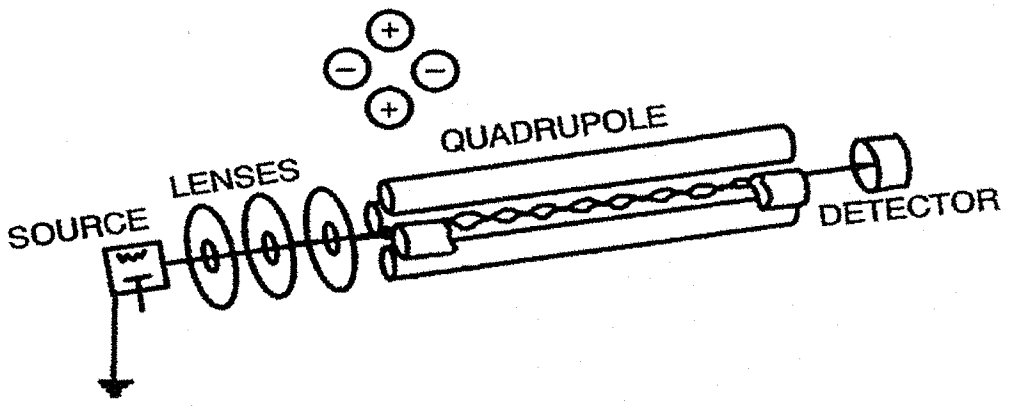


Figure 5.6 Schematic of a quadrupole system. This shows the ion trajectory from the source to the detector (Source: de Hoffmann, 1996)

Quadrupoles can also be used in tandem. Usually this is with three quads in series and is referred to as a triple quad. The first and third quads are used for scanning whilst the middle quad is used as a collision cell for further ion fragmentation. Tandem mass spectrometry is often used for enhanced structural elucidation.

Following selection of ions in the quads, the ion beam is detected and transformed into an electrical signal in the detector. Two main types of detector are frequently used, those that directly measure charges reaching the detector, like the photographic plate detector, and those that increase the intensity of the signal reaching it, like photomultiplier and array detectors. For the Trace MS^{TM} system a photomultiplier detector was used. The device allows for the detection of both positive and negative ions, although one or other is chosen at any one

time. When ions from the analyser hit the dynode, secondary electrons are produced which are accelerated towards a phosphorescent screen where they are converted into photons and detected by the photomultiplier. Amplification of the signal ranges from four to five orders of magnitude (de Hoffmann *et al.*, 1996).

Increased sensitivity can be achieved in the scanning mode by increasing counting time per mass (the scan time), or by reducing the mass range over which scanning occurs to ensure increased scanning frequency at a specific mass. Selected ion monitoring (SIM) can be used to obtain further specificity and sensitivity. This was the detection mode used by Krahn *et al.* (1993) and Røe Utvik and co-workers (1999). It is accomplished by selecting a pole voltage, allowing only a specific mass-to-charge ratio to pass on to the detector. Several of these voltages can be chosen to monitor a selection of specific masses. As no scanning is involved the detection time for each mass is increased thus resulting in sensitivity enhancement. There are typically two orders of magnitude increase in sensitivity on using SIM in preference to the scanning mode (Willoughby *et al.*, 1998).

In addition to quantification by high-resolution scanning or selected ion monitoring, the computer controlling the system can hold a database where the main peaks of known compounds are stored. Several ions of different mass-to-charge ratios are combined to form a fingerprint of the analyte (the spectrum) which is sufficiently reproducible to be matched to library spectra for identification purposes. This system is not foolproof as totally different compounds may have very similar spectra. This tool is therefore best used for confirmation purposes or to suggest possible rather than absolute identifications. Mass spectrometry is particularly important in this study for both the identification and quantification of the different components of liquid scintillant cocktails and products resultant from biodegradation.

5.3 UV-fluorescence spectroscopy (UV-FS) for bis-methylstyryl benzene (bis-MSB) analysis

UV-fluorescence spectroscopy is often used for hydrocarbon quantification and fingerprinting (Table 5.2). In this study it is utilised for fluor quantification of bis-MSB, one of the scintillators found in LS cocktails.

Table 5.2 Some examples of studies that have included fluorescence spectrometric analysis

Purpose of analysis	*Wavelength used*	*Reference*
Quantification of 2 – 5 ring aromatic hydrocarbon structures	250 – 500 nm	Boehm & Fiest (1982)
Pyrene quantification following HPLC separation	260 – 370 nm	Li *et al.* (1996)
Nonylphenol ethoxylate quantification	222 nm in hexane/water and octanol/water systems 275 nm in ethyl acetate/water systems	Menges *et al.* (1992)
PAH quantification	360 nm	Paalme *et al.* (1990)

5.3.1 UV-FS technique

Fluors have the capability of producing a coloured luminosity when light, especially in the violet and ultra-violet section of the wavelength spectrum is used to illuminate them. Identification and quantification of fluors can be achieved as each compound fluoresces at a given wavelength, hence response is proportional to analyte concentration.

A fluorescence spectrometer employs a xenon light source from which the light passes through a system of mirrors and prisms. A grating at the end of this sequence allows for selection of a particular wavelength (the excitation wavelength) that subsequently irradiates the sample placed in a quartz cuvette with a 1 cm path length. Molecules in the sample absorb the light, resulting in electrons moving to elevated orbitals. As they return to a lower orbital, energy is lost in the form of fluorescence, usually at about 25 nm longer than the excitation wavelength (Boehm & Fiest, 1982; Law *et al.*, 1988). This emitted light passes through an exact replica of gratings, mirrors and prisms to the excitation light, and on to the detector. A range of excitation wavelengths is

used to include the characteristic wavelengths of interest, which in the case of bis-MSB is 454 - 463 nm.

5.3.1 Bis-MSB analysis and method development

A great advantage of the UV-FS technique is that it is relatively simple and does not require complex sample preparation or lengthy analysis. However, problems found included large variations in the light source intensity, resulting in recorded fluorescence variations even on a day to day basis. A drift monitor and blanks were analysed with every set of samples to compensate for these changes. Detection limits for the apparatus were 0.5 ppm (± 0.1 ppm), however this was greatly dependent on the amount of excitation light.

Method development included standard calibrations of bis-MSB solutions at a range of concentrations (0 – 50 ppm). High correlation coefficients between fluorescence intensity and concentration were evident. This was particularly the case over a concentration range of 0 – 10 ppm (R^2 = 0.984).

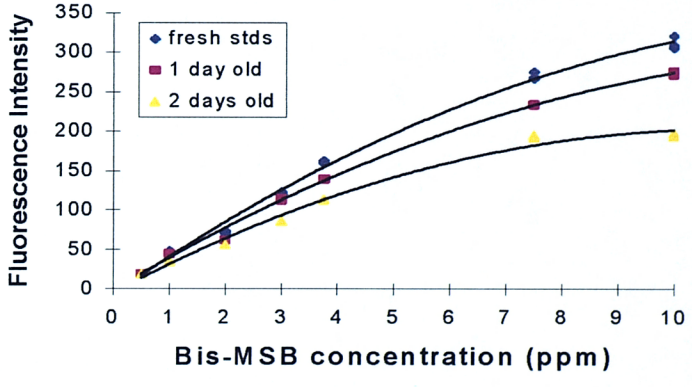


Figure 5.7 Changes in bis-MSB standard fluorescence response upon storage

The bis-MSB standards showed a decrease in fluorescence response with storage time which was most marked at high concentrations (Figure 5.7). This loss is likely to be as a result of surface activity of the bis-MSB with the storage vessel walls, and recommends both the use of fresh standards for each batch of analysis, and the rapid processing of samples following generation.

The addition of Gold Star™ to bis-MSB standards resulted in a substantial quenching of the measured fluorescence intensity of bis-MSB. Further analyses of the standard combined with PPO, BEE and DIPN individually showed that all of these analytes resulting in quenching of the signal to a greater or lesser extent. As variable levels of these interferences were present in the degradation samples, their relative quenching effect could not be quantified. Extensive sample preparation to separate bis-MSB from the other liquid scintillant cocktail constituents prior to analysis may have been a solution. However Wise and co-workers (1993) found that there was a good agreement between fluorimetry data and GC-MS quantification. Thus bis-MSB analysis by GC-MS was opted for instead.

5.4 GC-MS experiment procedure

The analysis of environmental samples involves two key steps, extraction and analysis. Method development and a review of the literature concerning each of these steps are dealt with in turn, followed by a discussion of quality assurance. Full details of the procedures are given in Appendix 4.

5.4.1 Solvent extraction

Extraction of hydrocarbon analytes from a sample matrix can be carried out by various different methods (Table 5.3).

The most conventional approach for solid samples is Soxhlet extraction. Here the apparatus is divided into the condensing tower, the extraction tube and the boiler. Samples are loaded into the extraction tube. The tube is connected to the condenser and placed on top of the boiler containing solvents. As the solvent heats up, it starts to evaporate, rinsing into the condensing tower, which is cooled by circulating cold water. The solvent condensate drips down onto the sample in the extraction tube, accumulating here and washing the sample. As the solvent building up, it reaches the upper opening of the siphon tube, whereby it is returned to the boiler. The solvent is then re-evaporated, while the extracted organics remain in the boiler.

Despite using relatively accessible and simple apparatus, this technique has limitations as it is both time-consuming and utilises large volumes of hazardous and toxic organic solvents (Chee *et al.*, 1996; Pensado *et al.*, 2000), e.g. pentane.

Table 5.3 Hydrocarbon extraction methods used for different applications

Extraction technique	*Compound(s) of interest*	*Reference*
Soxhlet extraction	• Alkylphenol ethoxylates • Dioxins • Aliphatic and polyaromatic hydrocarbons • Alkylphenols	• Menges *et al.* (1992) • Chang & Huang (1999) • Moreda *et al.* (1998) • Lye *et al.* (1999)
Liquid-liquid extraction	• Alkanes • Petroleum components • Nonylphenol ethoxylates • Monoaromatics • Hydrocarbons • Polyaromatic hydrocarbons	• Al-Hadhrami *et al.* (1997) • Boehm & Fiest (1982) • Shang *et al.* (1999b) • Rontani *et al.* (1986) • Venosa *et al.* (1993) • Røe Utvik *et al.* (1999); Boonchan *et al.* (1998)
Microwave-assisted extraction	• Polyaromatic hydrocarbons	• Pensado *et al.* (2000)
Supercritical fluid extraction	• Polyaromatic hydrocarbons	• Furton *et al.* (1993)
Solid-phase extraction	• Polyethylene glycols • Nonionic polyethoxylated surfactants • Nonylphenol ethoxylate biodegradation products • Polar organic pollutants	• Crescenzi *et al.* (1997) • Castillo *et al.* (1999) • Di Corcia *et al.* (1998a) • Schröder (1997)

Liquid-liquid extraction is the removal of a substance from a solution or a mixture of liquids by utilising its greater solubility in another liquid (Oxford University Press, 2001). It is less time-consuming than Soxhlet extraction but has similar solvent requirements. Several new techniques are beginning to replace these two conventional methods, including supercritical-fluid extraction, solid-phase extraction and microwave-assisted extraction. All these new techniques have much lower time and solvent requirements and yet show high extraction efficiencies and good reproducibilities (Camel, 2000; Davi & Gnudi, 1999; Ding & Fann, 2000; Hawthorne *et al.*, 2000; Levin *et al.*, 1993; Pensado *et al.*, 2000). However, the disadvantage to these methods is the requirement of

specialised apparatus, such as pressure-resistant reaction vessels and microwave systems. Due to time restrictions the availability of basic apparatus only, liquid-liquid extraction was chosen for the purpose of this study.

5.4.1.1 Choice of solvent

The solvent used in the process is greatly dependent on the compounds of interest and analytical technique to be used (Pensado *et al.*, 2000) (Table 5.4). The amount and volatility of solvent can also have an effect on the chromatography. For example Trevelin *et al.* (1992) showed that the use of xylene results in increased peak areas of late eluting peaks when compared to benzene. Furton *et al.* (1993) stated that the use of toluene and xylenes for the extraction and analysis of high molecular mass PAH gave enhanced signals of up to 100 times in comparison to other solvents. In addition to analytical issues, safety aspects have to be considered, including toxicity and fire risk (Howard & Statham, 1993).

Table 5.4 Solvents used for different compounds similar to those used in this study

Analyte of interest	*Solvent used*	*Reference*
Nonylphenol ethoxylate	Ethyl acetate	Menges *et al.* (1992)
Monoaromatics	Chloroform	Rontani *et al.* (1986)
Various hydrocarbons	Hexane	Venosa *et al.* (1993)
Lipids	Dichloromethane	Al-Lihaibi & Ghazi (1997)
Petroleum constituents	Chloroform	Walker *et al.* (1976)
Petroleum hydrocarbons in water	Dichloromethane	Boehm & Fiest (1982)
PAH	Dichloromethane	Røe Utvik *et al.* (1999)
PAH	Hexane, dichloromethane and chloroform	Furton *et al.* (1993)
Hydrocarbons	Dichloromethane : methanol (2:1 v/v)	Al-Lihaibi & Ghazi (1997)
Alkanes	Dichloromethane	Al-Hadhrami *et al.* (1997)
Nonylphenol ethoxylates	Hexane : acetone (1:1 v/v)	Shang *et al.* (1999b)

Dichloromethane was initially chosen for this study, as this was readily available. Subsequent tests proved it to be an effective solvent for this purpose. Dichloromethane is considered a powerful solvent and when combined with

acetone is capable of extracting all of the organics from a sample (Prince, 1993). D_8-naphthalene standards were made up in two different solvents (dichloromethane, and dichloromethane:hexane mix, 1:1 v/v) to determine if the different volatilities affected analytical reproducibility. No significant difference was found between the two solvents, with a relative standard deviation of 2.5 and 2.9 % respectively. However, peak areas detected for the mixed solvent were significantly lower, and therefore dichloromethane (DCM) was chosen for liquid-liquid extraction purposes.

5.4.1.2 Liquid-liquid extraction procedure

The procedure used in this study involved the combination of equal volumes of aqueous bioreactor sample and dichloromethane. The combination was mixed thoroughly for a specific period of time and left to separate. A specific fraction of the bottom, organic layer was aspirated into a clean glass liquid scintillant vial and the remainder was removed to waste. This procedure was repeated several times, combining the set organic fraction from each extract for analysis (for details see Appendix 4).

Gold Star™ (GS) solutions of 1–30 % in Milli-Q™ water were extracted. Aspiration of the organic fraction proved both variable and problematic for concentrations at and above 10 % as separation into the two phases (organic and aqueous) did not readily occur. This separation was hampered by the presence of surfactants in the GS resulting in emulsification. An experiment was conducted to determine the optimum method for suppressing the emulsifier action. Several different salt solutions (NaCl) and dilute hydrochloric acid were added to separate the organic and aqueous phases of 10 % GS solutions extracted with DCM. 500 µL (10 drops) of 1.2 M $HCl_{(aq)}$ was found to be most effective, therefore this was added at the first extraction stage for each sample. Extractions of GS solutions at different concentrations indicated that effective separation occurred with the hydrochloric acid addition and that there was no significant difference between the final volumes of the two layers, suggesting that the either the emulsifier action had been overcome, and/or acidification had caused a shift in the reaction equilibrium resulting in a more efficient extraction of the organic fraction.

A similar partition coefficient experiment was conducted by Menges *et al.* (1992) for the analysis of nonylphenol ethoxylates (NPEs). They found that when high concentrations of NPEs were extracted in octanol/water shake-flask experiments a persistent emulsion layer was formed, as observed in this study, albeit with a different solvent and a mixed sample. Menges and co-workers also showed that a 'salting-out' effect of both the solvent and analyte occurred when NaCl at a concentration of 2.0 M was added to the mixture. The salt addition effectively resulted in an increase in the partition coefficient and extraction efficiency. Boonchan *et al.* (1998), Sumino (1977) and Susarla *et al.* (1998) also used hydrochloric acid for this purpose.

Extraction of a degradation sample was carried out in duplicate, with and without the addition of hydrochloric acid, to determine whether or not this affected the chemistry of the analytes. It was shown that although sample spectra and profiles did not differ, there was a significant improvement in extraction efficiency as a result of the acid stage inclusion (5 *vs.* 100 % extraction efficiency). This pre-extraction acidification step acts to perturb the emulsion equilibrium thus aiding effective extraction.

Five sequential extractions were carried out for 0.5 and 1 % GS solutions. These indicated that there was no further extraction occurring for the 4^{th} and 5^{th} extracts, thus only three sequential extractions were carried out. GC-MS analysis at each stage of the extraction procedure showed that there is no preferential extraction of specific analytes as ratios between them were constant throughout. The cumulative extraction efficiency was 84 %, 92 % and 95 % for the first, second and third extractions respectively. This is comparable to the work of Bolz *et al.* (2000) who found recovery rates of 80 – 100 % following the use of 5 g/L of salt to increase the partition coefficient. The authors also noted that at a salt concentration of 10 g/L recoveries were reduced, suggesting that there is an optimum concentration to ensure maximum recovery. The recovery found in this study is also comparable with that found by Li *et al.* (1996), Marcomini & Zanette (1996), Marcomini *et al.* (1998) and Schuhmacher *et al.* (1999), and higher than several others. If further recovery

and precision had been required, a larger and therefore more representative sample could have been used (Evans *et al.*, 1997). Extraction reproducibility was assessed by numerous replicate extractions of standards and samples. Extraction reproducibility, following internal standard corrections, had a relative standard deviation of 3.8 – 6.5 % for BEE, DIPN and PPO (an average based on triplicate extractions and experiments).

Analysis of extracted samples at a variety of GS concentrations ranging from 0.1 to 10% showed that there is no marked difference in final extraction efficiency with initial concentration. This means that all degradation samples, regardless of their concentration, can be processed, analysed, compared and interpreted in the same manner.

Several authors have used a clean up stage following extraction. The aim of this step is to remove any possible interferences such as alkanes and fatty acids from the sample that may limit GC separations and MS library matches. In addition to this purification step, the process also allows for separation of the fractions for analysis, e.g. polar and saturated hydrocarbons. Adsorption chromatography by means of a packed fluorisil, silica or alumina column is often used. Amongst others, Rontani *et al.* (1986) used a silica-gel column to remove asphaltenes and separate hydrocarbon fractions. Nondek *et al.* (1993) used a DNAP-silica column to remove a wide range of interfering pollutants from river sediment samples being analysed for polyaromatic hydrocarbon concentrations. This solid-phase extraction stage was not used in this study, as it is not always clear which compounds the process will remove. With an attempt to monitor unknown biodegradation products these very compounds may inadvertently be removed prior to analysis.

5.4.1.3 Sample dilutions

All samples were diluted 100-fold prior to analysis to prevent saturation of the detector when the sensitivity of the mass spectrometer was optimised. This dilution step could have been carried out on the sample prior to extraction or on the extract just prior to analysis. Initial separation problems of the degradation sample-solvent mixture, despite the addition of dilute hydrochloric acid, were

overcome by advance dilution of the bulk sample by a factor of 2.5 with Milli-Q™ water. This resulted in a clear separation of the two fractions, allowing for effective and reproducible extractions. By combining the three extracts and an equal volume of internal standard, overall dilution of 1 in 10 was achieved. A further ten-fold dilution was carried out immediately prior to analysis. Full details of sample preparation procedures are given in Appendix 4.

5.4.2 GC-MS operation

The ThermoFinnigan Trace-MS 2000 system used for the majority of the GC-MS analysis was a manual injection, split/splitless system hence certain parameters such as injection speed were set. As for all analytical equipment, sensitivity is strongly increased by optimising conditions. Details of operating conditions are given in Appendix 4.

5.4.2.1 Injector parameters

Hot needle splitless injection was chosen to minimise variable analyte losses and maximise transfer onto the column. A split event was introduced after 1 minute to purge any remaining sample out of the vent and prevent it passing on to the column which would have had the effect of reducing peak resolution. It was decided that should the sample be sufficiently concentrated to saturate the mass spectrometry detector, then a further dilution step would be included in preference to split injection. A 1 µL sample volume at a 100-fold dilution provided the required peak response for all the degradation samples.

A further consideration was whether to use the injector in an isothermal mode or to utilise the temperature ramping facility to optimise injection reproducibility. A 50 ppm standard containing the majority of the key analytes in a known concentration ratio was analysed five times under both conditions. The isothermal injector temperature was 250 °C, and the ramped injector programme used was 50–300 °C at 50 °C/min. Both procedures involved a GC oven temperature ramp for the column and sample following injection. The temperature programmed injections showed a slightly lower peak area, and there was little difference between the relative standard deviations of the two injection types (4.4 % as opposed to 5.5 % for the isothermal injections). Due to

small difference between the injection methods, isothermal injection was chosen for this study because it was the standard method used on the apparatus.

5.4.2.2 Gas chromatography parameters

A ramped oven temperature programme over a large range was required to include and separate the wide variety of compounds of interest. Following several analyses with different temperature ramp rates, and starting and finishing temperatures, it was found that a single ramp from 50 - 300 °C at a rate of 10 °C/min. resulted in sufficient peak resolution and a relatively short analysis duration. An initial 3 minute isothermal period was required to allow the solvent front to pass through the system prior to elution of the key analytes. A further 7 minute isothermal period following the temperature ramp allowed for any remaining compounds to be eluted from the column, preventing contamination and carryover.

The column used was a PE 5-MS fused silica capillary column (30 m x 0.25 mm internal diameter, and a film thickness of 0.25 µm). This is a standard 5 % phenyl equivalent bonded phase column used for GC-MS analysis of hydrocarbons and has also been used for several other studies including Al-Hadhrami *et al.* (1997), Chee *et al.* (1996), Lye *et al.* (1999), Moreda *et al.* (1998) and Schuhmacher *et al.* (1999). Helium gas was used as the carrier and the transfer line temperature was held at 250 °C.

5.4.2.3 Mass spectrometric parameters

A standard source temperature of 250 °C was used for the mass spectrometer. This is comparable to that used in other studies like Davi & Gnudi (1999).

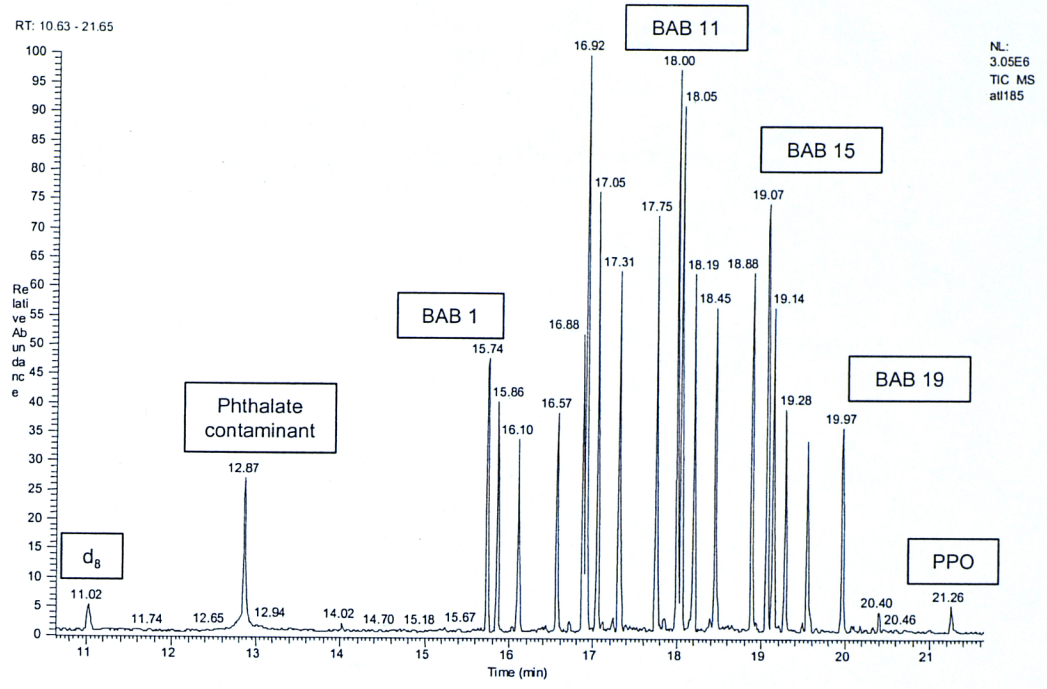
Standards of the main compounds of interest were analysed by GC-MS. The data allowed for confirmation of analyte identification and determination of the related analyte retention times. If all conditions of the analytical procedure, particularly the oven ramping rate, are maintained then retention times can be used to earmark a compound (Table 5.5 and Figure 5.8). Retention time

reproducibility was high, with a relative standard deviation of 0.1 % (Appendix 5).

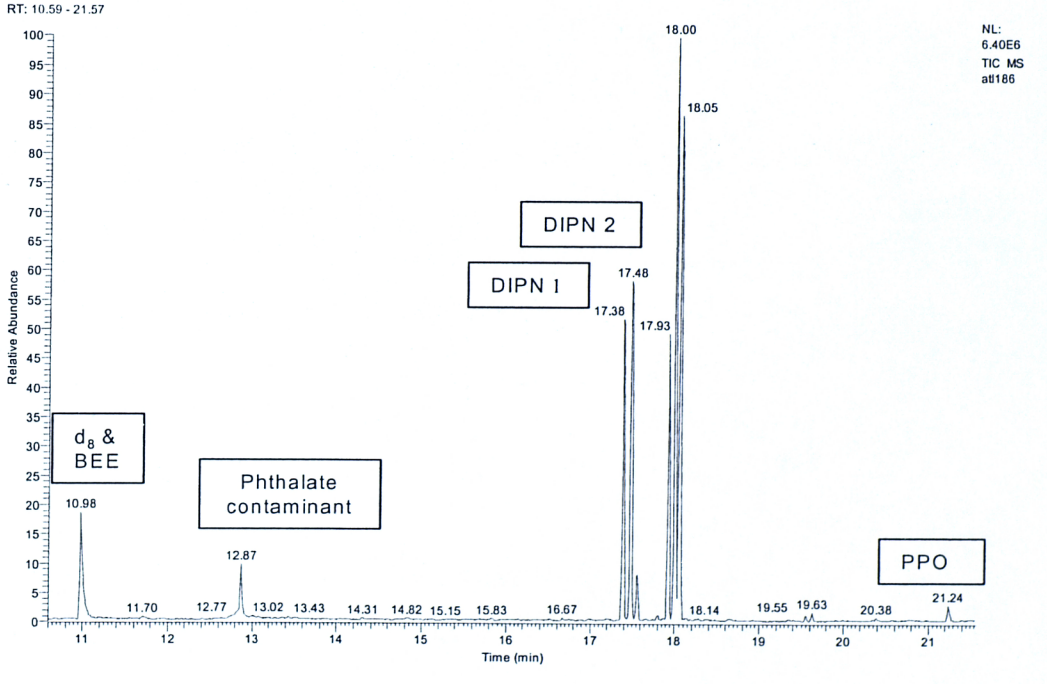
Commercially available di-isopropyl naphthalene is a group of position-isomeric di-isopropyl naphthalenes. This was evident from the analysis that showed a cluster of peaks at a retention time of about 17.4 mins (Figure 5.8(b)). It is only the two main peaks that are monitored for this study. The principle components of the commercial di-isopropyl naphthalene mixture are 2,6- and 2,7- alkylated isomers (Höke & Zellerhoff, 1998) and therefore it is likely that the two peaks chosen correspond to these isomers.

Table 5.5 Retention times and abbreviated names of the compounds of interest under standard operating conditions used in this study

Compound	*Abbreviated name*	*Retention time (minutes)*
Butoxyethoxy ethanol	BEE	10.98
D_8-naphthalene	D_8-naphthalene	10.99
1-butylhexyl benzene	BAB 1	15.72
1-propylheptyl benzene	BAB 2	15.85
1-ethyloctyl benzene	BAB 3	16.09
1-methylnonyl benzene	BAB 4	16.55
1-propyloctyl benzene	BAB 7	17.03
1-ethylnonyl benzene	BAB 8	17.29
Di-ispropyl naphthalene isomer 1	DIPN 1	17.38
Di-ispropyl naphthalene isomer 2	DIPN 2	17.47
1-methyldecyl benzene	BAB 9	17.74
1-pentylhepty benzene	BAB 10	17.98
1-butyloctyl benzene	BAB 11	18.03
1-propylnonyl benzene	BAB 12	18.18
1-ethyldecyl benzene	BAB 13	18.44
1-methylundecyl benzene	BAB 14	18.86
1-pentyloctyl benzene	BAB 15	19.05
1-butylnonyl benzene	BAB 16	19.12
1-propyldecyl benzene	BAB 17	19.26
1-ethylundecyl benzene	BAB 18	19.52
1-methyldodecyl benzene	BAB 19	19.94
2,5-diphenyl oxazole	PPO	21.22
Bis-methylstyryl benzene	Bis-MSB	32.17



(a) EcoSafe™ spectrum. Not all peaks of interest are labelled due to space restrictions



(**b) Gold Star™ spectrum**. The cluster of peaks at about 18 mins. are other DIPN isomers that were quantified in this study

Figure 5.8 Typical spectra showing the compounds of interest, under standard operating conditions. Note: Phthalate is a common contaminant in gas chromatography, often resulting from injector septum break down.

Initial use of total ion current (TIC) for sample analysis showed that all peaks could be detected in this acquisition mode. Peak area rather than peak height was used for quantification in all cases. Peak areas of the majority of the compounds of interest were large enough for them to be distinguished from background noise, yet small enough to prevent detector saturation. Saturation of the detector is indicated by a characteristic 'flat-topped' rather than Gaussian peak shape, and results in poor reproducibility of the data. Bis-MSB peaks were very small in comparison to other analyte peaks and therefore any changes in peak size were difficult to detect. It was therefore decided that further analysis of less dilute samples would be undertaken for bis-MSB quantification if time allowed it. Detection limits of the technique are 1 – 10 ng on-column when analysis is carried out for unknown compounds in the full scan mode (Kaye, 2001, *pers comm.*). This is comparable with Sumino (1977) who found limits of 0.1 ng for DIPN determination by GC-MS and Crescenzi *et al.* (1995b) who had detection limits of 0.02 ng per nonylphenol ethoxylate homologue.

A distinct advantage of the use of GC-MS as opposed to simple GC was that despite the co-elution of BEE and d_8-naphthalene post-processing allowed for the differentiation of peaks using their specific spectrum for identification purposes. This allowed them to be reproducibly resolved and quantified.

In order to obtain reliable quantification, the fastest possible scan rate is required. This can best be achieved using the selected ion monitoring (SIM) mode (Marcomini & Zanette, 1996). However, as the aims of this study included monitoring of any biodegradation products, SIM would require the specification of specific ions for monitoring and therefore would not detect any unknown products. As reproducibility, sensitivity and specificity was sufficient in the TIC mode, this acquisition mode was utilised. Replicate analysis of a testmix, with no internal standard corrections and manual injection gave a peak area reproducibility of 1.5 – 5.4 % relative standard deviation for 5 individual compound peaks (n = 5). Table 5.6 provides an overview of some of the mass spectrometric scanning parameters used for different applications.

Table 5.6 Scan parameters used for different studies, bold indicates values used in this work

Scan range (Da)	*Scan rate (scans/sec)*	*SIM carried out?*	*Reference*
35 – 350	1.5	No	Davi & Gnudi (1999)
45 – 500		Yes	Lye *et al.* (1999)
35 – 500	1	Yes	Law *et al.* (1988)
50 – 400	1.5	No	Maki *et al.* (1996)
50 - 590	**2**	**No**	**This study**
50 – 1000	0.3	No	Scullion *et al.* (1996)

5.4.3 Quality assurance and quality control

Samples were analysed in batches of 15 – 20 samples. Prior to any sample analysis a 1 µL aliquot of standard SGE testmix C was analysed to determine the sensitivity of the apparatus. The standard GS-MS procedure was used with peak area of the naphthalene peak (retention time = 11.05 min.) being monitored (Figure 5.9). A slight decrease with time is evident superimposed by several events of sensitivity loss. The areas given are not internal standard corrected and therefore are subject to analysis variations including variable injection volumes. As can be noted from the figure, rapid sensitivity loss resulted in remedial action being taken. Action possibilities included changing the injector septum, cleaning the source or raising the injector and oven temperature to near the operational maximum for a set period of time, e.g. overnight, in an attempt to 'bake off' the contaminant causing an increased baseline level and reduced sensitivity.

Extracted samples an solvent blanks were randomly selected for triplicate analysis ofr sample reproducibility and carry-over. All samples were also analysed randomly to prevent any systematic errors resulting in artificial trends being superimposed on the data.

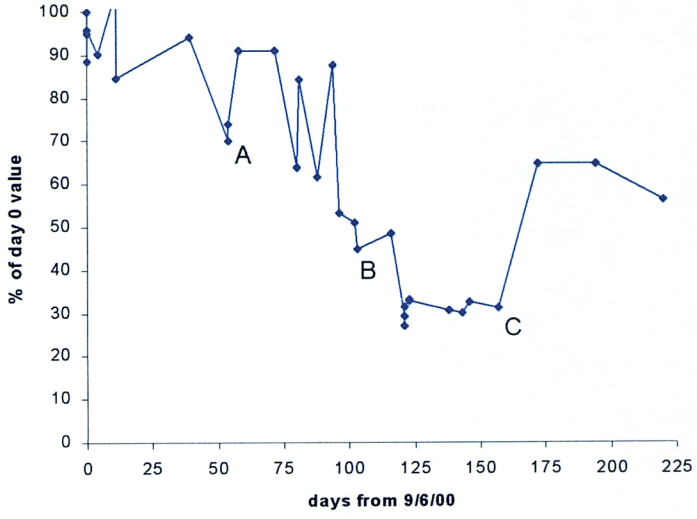


Figure 5.9 Changes in testmix naphthalene peak area with time. Day 0 is 9/6/00, the date from which sensitivity measurements were conducted.
A – injector septum changed
B – injector liner and septum changed
C – injector septum and all ferrules changed

Despite retention time reproducibility being high, peak areas showed some variations. The internal standard method uses the comparison of the signal intensity corresponding to the compound of interest with that of the reference compound (internal standard). If an internal standard is chosen with chemical and physical properties comparable to the other analytes, it is assumed that the two will undergo the same losses in the extraction steps, and the same errors during GC-MS analysis, therefore their ratio remains constant during the procedure. Knowing the initial internal standard concentration allows for corrections to be made for losses and errors (de Hoffmann *et al.*, 1996). The internal standard can be added to the sample prior to extraction to determine procedural errors of the entire process, or just prior to analysis for analytical variations.

The correction using d_8-naphthalene as an internal standard resulted in a great improvement of peak area reproducibility. On conducting the extraction method development, it became evident that during sequential extractions d_8-naphthalene was not extracted in the same ratios as the other analytes. It was thus only added prior to analysis to monitor analytical variability and be used for

correction purposes, as in the studies by Bolz *et al.* (2000) and Marcomini & Zanette (1996). Internal standards have been used to monitor and correct for extraction variability by Boonchan *et al.* (1998) who used 2,3-benzo[*b*]fluorene, and Susarla *et al.* (1998) who used di-isopropyl naphthalene for the purpose. The d_8-naphthalene peak area was determined following each sample analysis. If this area exceeded 150,000 the next sample was analysed immediately, whereas remedial action was undertaken to restore sensitivity if this threshold value was not attained. Chee *et al.* (1996), Susarla *et al.* (1998), Thomas *et al.* (1999) and Venosa *et al.* (1993) also used d8-naphthalene for internal standard correction purposes. External standard calibrations were not necessary for this study, as relative rather than actual concentrations were required to draw conclusions on whether or not degradation was occurring.

The Excalibur™ software used automatically processed raw sample data to identify peaks of interest on the basis of key ions in sample spectra supplied on setting up the processing procedure. Following identification and earmarking of the peaks, their relative position to the internal standard was checked to avoid two compounds with similar spectra being identified as a particular analyte. The peaks were then automatically integrated using the parameters specified in the method (for details see Appendix 4). This method allowed for the co-eluting peaks of BEE and d_8-naphthalene to be differentiated and integrated separately. The quantification option of the software was used to manually select the baseline and start/finish point of each peak. This was to ensure that the entire peak was included regardless of slight changes in retention time and peak shape.

In summary therefore, the sample was diluted 2.5-fold prior to extraction. Triplicate liquid-liquid extractions were carried out using dichloromethane. Hydrochloric acid was added to quash the emulsifier action of the liquid scintillant cocktails and aid phase separation. The three extraction aliquots were combined, and an equal volume of d_8-naphthalene standard was added, resulting in a final dilution factor of 10. A further 10-fold dilution was carried out just prior to analysis by GC-MS.

Analysis was using an isothermal splitless injection GC-MS system. Peak areas were used for quantification purposes and the internal standard was used to normalise the peaks for injection and analysis variations.

5.5 GC-MS results

This section deals with each of the compounds of interest, outlining the degradation profiles, abiotic losses and impact on biodegradation of the simultaneous presence of other carbon sources. A brief description of compound biodegradation in Gold Star™ and EcoSafe™ is followed by an assessment of biodegradation process reproducibility. Discussion and conclusions are given in Section 5.6.

All the data has been corrected using the d_8-naphthalene internal standard peak. This compensates for sample injection and analysis variations. However this standard does not compensate for variable mixing in the bioreactor. As a result, when separate sub-samples of the same bioreactor sample are analysed some variation in the data is shown (Figure 5.10 and 5.11). All graphs for BEE, DIPN and PPO show these variations.

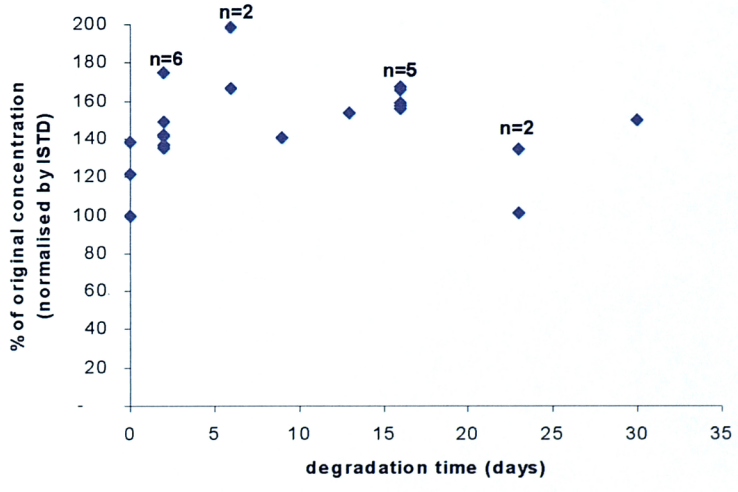


Figure 5.10 Complete DIPN peak 1 data set for concentration changes in a bioreactor containing Gold Star™, with activated sludge. The number of separate samples analysed are indicated in some cases. Marked data variations are evident.

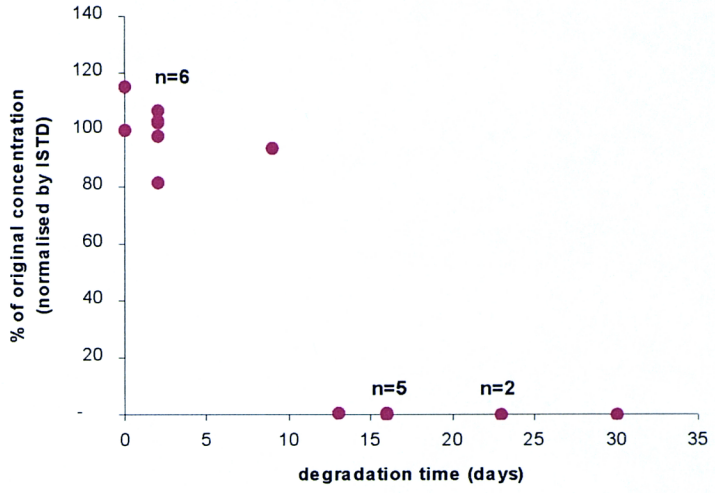


Figure 5.11 Complete PPO data set for concentration changes in a bioreactor containing Gold Star™, with activated sludge. The number of separate samples analysed are indicated in some cases. Marked data variations are evident

All the data given in this section for BAB are not only corrected for analytical variations by a d_8-naphthalene internal standard correction, but also by a further correction to account for bioreactor heterogeneity. Ratioing of the internal standard corrected peak areas to the BAB 15 peak (C_{19} pentyl- primary chain isomer) was used to compensate for non-homogenous mixing of the bioreactor. As this isomer showed no losses with time, it was the ideal choice as an internally conserved standard, in the same way that hopane is sometimes used in crude oil biodegradation studies. Any changes in the ratio between this standard isomer and peak X therefore indicates that peak X is undergoing losses of some kind.

This second correction for the BAB data had a big influence on normalising the data (Figure 5.12), facilitating interpretation. The BEE, DIPN, and PPO graphs given are therefore more difficult to explain and only trends rather than actual values can be used for interpretation purposes. Further data given in this result chapter is based on averages of the multiple sample data points.

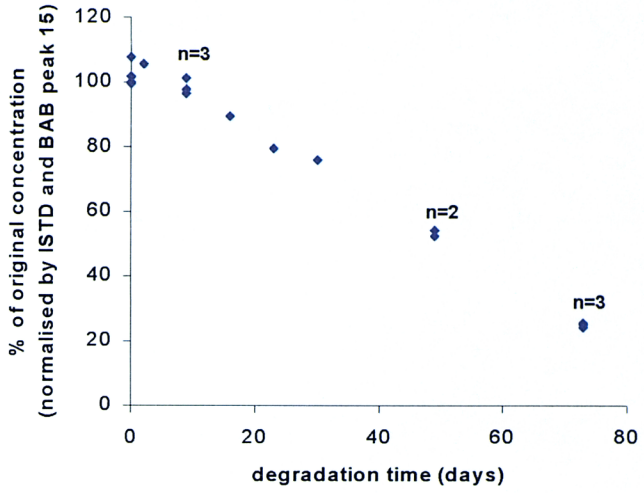


Figure 5.12 Complete BAB isomer 1 data set for concentration changes in a bioreactor containing BAB only, with activated sludge. The number of separate samples analysed are indicated in some cases. Only very small data variations are evident

The abiotic loss data has been classified according to the assumed loss mechanisms involved. As described in Chapter 3, biodegradation experiments were set-up with increasing number of possible loss mechanisms, thus resulting in cumulative losses. The mechanisms included losses due to sorption, airborne contaminating microbes and a combined category of 'other', including photo-oxidation and volatilisation. It should be noted that the mechanisms themselves were not actually measured, but rather that any losses were attributed on the basis of relative contribution to the cumulative total.

5.5.1 Butoxyethoxy ethanol (BEE)

BEE is the co-solvent present in Gold Star™ (GS). Losses were evident for this compound, particularly when present with activated sludge as the sole carbon source (BEE 1) or in Gold Star™ (GS 1) (Figure 5.13). The data showed total losses by day 9 of the biodegradation experiment. It is evident that the presence of other potential carbon sources, as is the case when BEE is present in GS (GS 1), did not markedly affect the extent of loss of this compound.

Some losses were also evident in the bioreactor containing no activated sludge (BEE control). This indicates that abiotic losses are also affecting the BEE concentration. Further data regarding the abiotic loss types occurring, indicated

that photo-oxidation/volatilisation and sorption play a part in the abiotic losses incurred.

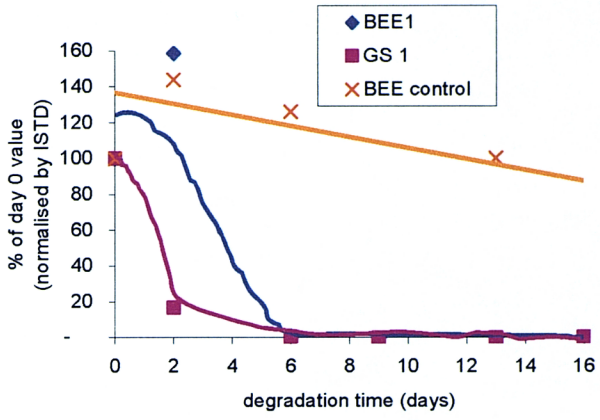


Figure 5.13 BEE losses in different carbon source mixtures. All values on the y-axis are given as % of day 0 values when the peak areas are ratioed against the internal standard (ISTD) peak

The figure shows that degradation losses were not constant with time. The period prior to the onset of losses is termed the lag phase. Following the period of maximum losses there is a time where no further concentration changes occur. No lag phase is apparent in this BEE data.

Outcomes:

- **BEE losses occurring - yes**
- **Abiotic losses mechanisms involved - photo-oxidation/volatilisation and sorption,**
- **Dominant loss mechanism – biotic,**
- **Lag phase apparent – no, and**
- **Losses affected by the presence of other compounds – no.**

5.5.2 2,5-diphenyl oxazole (PPO)

PPO is used as a primary fluor in both GoldStar™ (GS) and EcoSafe™ (ES). PPO concentration losses with time occurred when the compound was present as a sole carbon source (PPO 1) and as part of the liquid scintillant cocktail GS (GS 1) (Figure 5.14). Further data indicated that this loss was also true when the compound was present in a bioreactor containing ES.

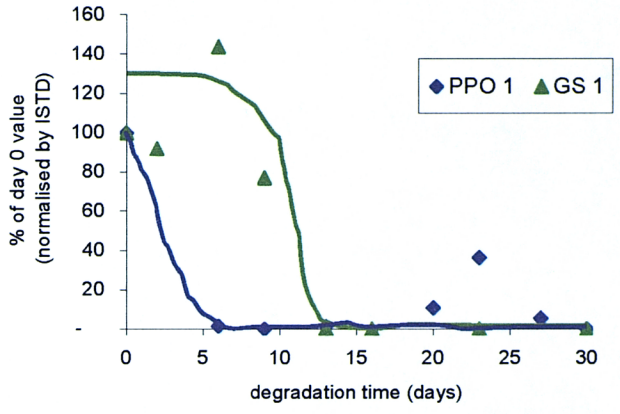


Figure 5.14 Losses of PPO in different carbon source mixtures. All values on the y-axis are given as % of day 0 values when the peak areas are ratioed against the internal standard (ISTD) peak

As for BEE, PPO also shows losses in analyte concentration with time, with total loss occurring in the bioreactors containing activated sludge. Although these losses are apparent, due to normalisation problems it is difficult to determine exactly at which point they occur. Further biodegradation experiments indicated that the principle abiotic loss mechanisms involved were photo-oxidation/volatilisation. The losses were greatest when a viable activated sludge biomass was used suggesting that both biotic and abiotic losses are affecting PPO concentration.

No lag phase is indicated for the bioreactor containing solely PPO (PPO 1), however a 9 day lag phase is present for the GS 1 bioreactor. In addition, PPO degradation rates are dependent on the other analytes present in the mixture as indicated by the delayed onset of losses in the GS 1 bioreactor.

Outcomes:

- **PPO losses occurring – yes**
- **Abiotic mechanisms involved – photo-oxidation/volatilisation,**
- **Dominant loss mechanism – neither,**
- **Lag phase apparent – only is GS and ES bioreactors, and**
- **Losses affected by the presence of other compounds - yes**

5.5.3 Di-isopropyl naphthalene (DIPN)

DIPN is used in GS as a primary solvent, and as such it is the major component of this liquid scintillant cocktail. In the case of this study, two isomers were monitored, namely DIPN peak 1 and DIPN peak 2. These two isomers show a consistent ratio in the samples, therefore with an elevated concentration of one isomer, the other peak area is correspondingly high (Figure 5.15). Figure 5.10 suggests that the variations in concentration in Figure 5.15 can be attributed to sampling variability. Thus there are no substantial losses evident for this compound. As there are no losses when there is a viable biomass present in the bioreactor, this indicates that both abiotic and biotic losses are negligible. Further biodegradation experiment data showed that small abiotic losses could be attributed to sorption.

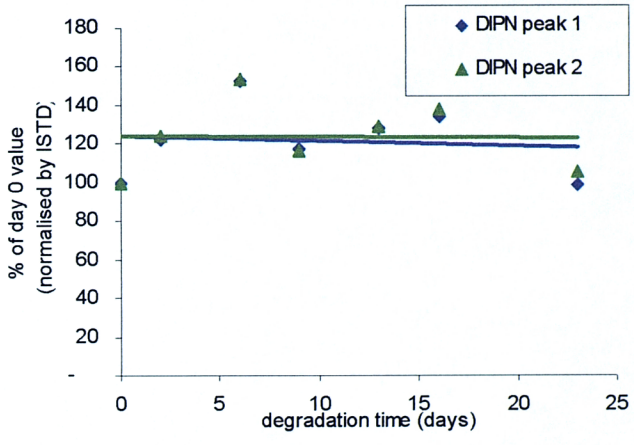


Figure 5.15 Concentration change with time of DIPN peaks 1 and 2 in a bioreactor containing activated sludge (AS), and DIPN as the sole carbon source. All values on the y-axis are given as % of day 0 values when the peak areas are ratioed against the internal standard (ISTD) peak. Fluctuations in the % present is solely due to bioreactor heterogeneity rather than actual concentration changes

Due to no evidence of losses with time no lag phase is apparent. No marked impact on losses was shown when other compounds were present, as in the case of the bioreactor containing GS (GS 1) (Figure 5.16).

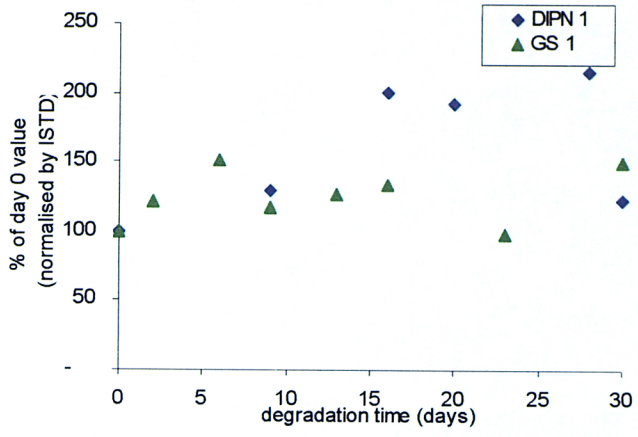


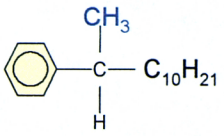
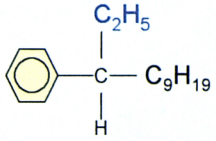
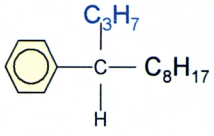
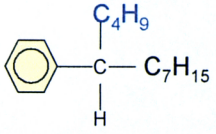
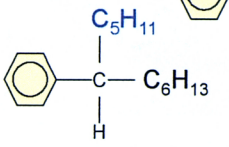
Figure 5.16 DIPN 1 losses in bioreactors with DIPN as a sole carbon source (DIPN 1) and as part of GS (GS 1). All values on the y-axis are given as % of day 0 values when the peak areas are ratioed against the internal standard (ISTD) peak. Any variations can be attributed to bioreactor heterogeneity resulting in fluctuations in the data

Outcomes:

- **DIPN losses occurring – minimal, if any,**
- **Abiotic loss mechanisms involved – sorption,**
- **Dominant loss mechanism – abiotic,**
- **Lag phase apparent – no, and**
- **Losses affected by the presence of other compounds – no.**

5.5.4 Branched alkyl benzenes (BAB)

The BAB mixture added to ES as a primary solvent consists of different molecular masses, ranging from C_{16} to C_{19}, each with a variety of different isomers of 1, 2, 3, 4 and 5 carbon atoms on the primary aliphatic chain (methyl-, ethyl-, propyl-, butyl- and pentyl- isomers respectively). For full details of the names of the 17 identified peaks see Table 5.5. An example of the naming system and isomer structures for the C_{18} isomers is given below. The blue section of the name refers to the shorter aliphatic chain. For the purposes of this study this is known as the primary aliphatic chain and it is this that is principally used to group the isomers.

$C_{18}H_{30}$	methyl undecyl benzene	
$C_{18}H_{30}$	ethyl decyl benzene	
$C_{18}H_{30}$	propyl nonyl benzene	
$C_{18}H_{30}$	butyl octyl benzene	
$C_{18}H_{30}$	pentyl heptyl benzene	

A distinct trend of increasing compound losses with decreasing primary chain length, with a given number of carbon atoms, is evident (Figure 5.17). Almost total compound losses are evident for the methyl-isomer, whereas the pentyl-isomer shows losses of only about 10 % within the 73 days of the experiment. The graph also shows a lag phase for all the isomers.

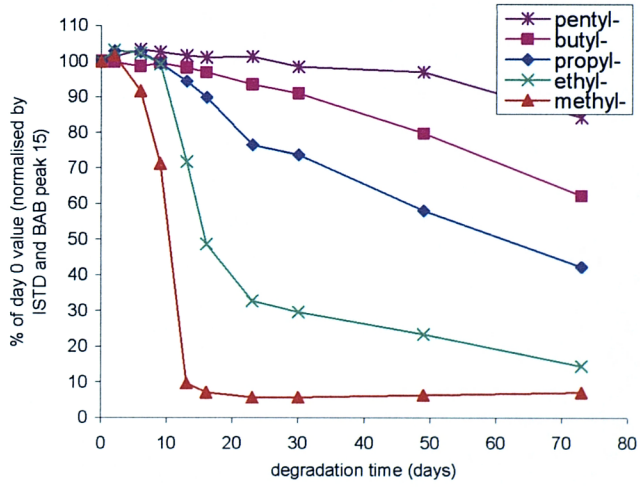


Figure 5.17 Concentration changes of C_{18} isomers in a bioreactor containing EcoSafe™ and a viable biomass source. This data incorporates both an internal correction using the d_8-naphthalene peak, and ratioing to the C_{19} pentyl- isomer (BAB peak 15)

When a standard primary chain length is chosen, and the secondary chain length is varied, hence altering the total number of carbon atoms present and therefore the molecular mass, a relatively small impact was evident (Figure

5.18). A lag phase is evident in the first 9 days of the experiment, with little, if any change in compound concentration. Following this period losses commence. Greatest losses are apparent for the compounds with a longer secondary chain length and therefore a higher molecular mass. However, by day 23 the extent of loss is equal for all compounds shown.

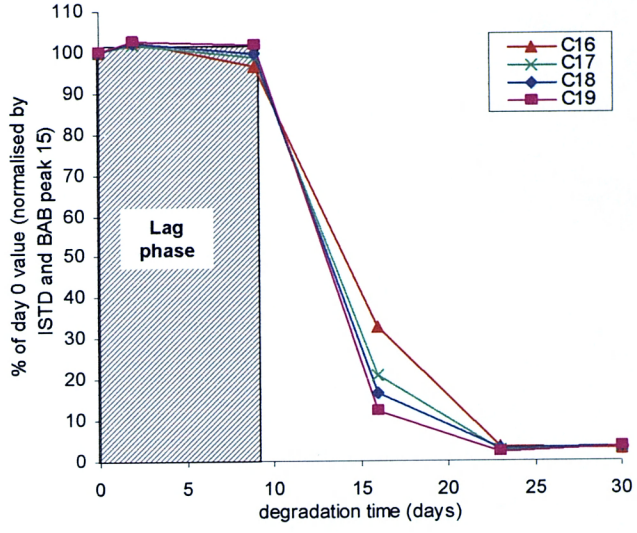


Figure 5.18 Concentration loss of ethyl- isomers of different molecular masses (all have two carbon atoms in the primary chain length). Bioreactors contain BAB as the sole compound and a viable biomass. This data incorporates both an internal correction using the d_8-naphthalene peak, and ratioing to the C_{19} pentyl- isomer (BAB peak 15). The 9-day lag phase is shown.

In the remainder of this BAB data section, C_{18} is used as standard to describe any trends with variations in primary chain length. To reduce the number of figures, details regarding molecular mass variations are described only.

The presence of a viable biomass in the form of activated sludge (AS) has a distinctive impact on compound concentration (Figure 5.19). The control bioreactor (BAB control) does not contain AS and is therefore only subject to abiotic losses including photo-oxidation/volatilisation and those due to contaminating airborne microorganisms. These losses are minimal. However, there is a large contribution from the combined mechanisms of sorption and biodegradation (BAB rig 1), with 95 % losses by day 23. A lag phase is evident, lasting 9 days. Beyond this point the cumulative losses in BAB rig 1 commence resulting in a very rapid loss of compound concentration.

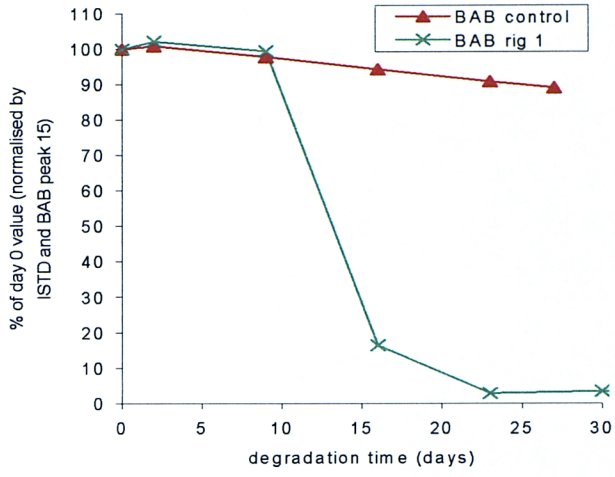


Figure 5.19 Concentration changes with time of C_{18} ethyl- isomer in bioreactors with (BAB rig 1) and without (BAB control) activated sludge. This data incorporates both an internal correction using the d_8-naphthalene peak, and ratioing to the C_{19} pentyl- isomer (BAB peak 15)

The distinct difference between bioreactors containing AS and those without is also evident for all isomers showing any losses. Further experiments provided details of the abiotic loss mechanisms involved. As in the case of combined abiotic and biotic losses shown in Figure 5.17, the extent of abiotic loss is also a function of the primary chain length (Figure 5.20), with decreased abiotic losses with increasing chain length. A 9-day lag phase is apparent for all the isomers showing discernible losses.

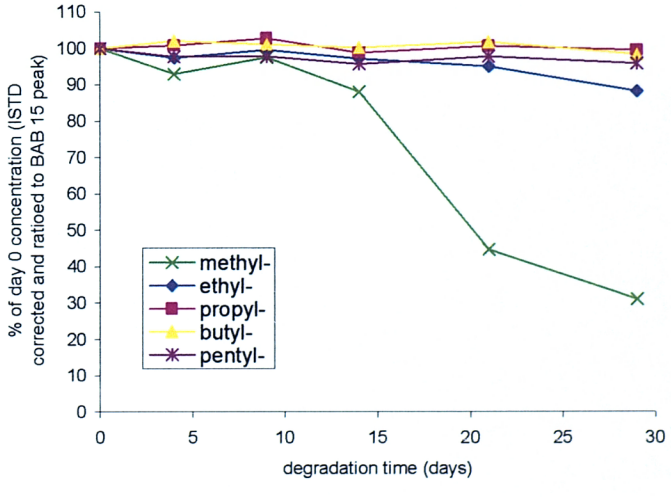


Figure 5.20 Concentration changes of C_{18} isomers as a result of all abiotic losses, showing relationship with primary chain length. This data incorporates both an internal correction using the d_8-naphthalene peak, and ratioing to the C_{19} pentyl- isomer (BAB peak 15)

However, the abiotic losses shown may be as a result of any of the mechanisms tested, including photo-oxidation, volatilisation and sorption. It is evident that each mechanism contributes to the losses found (Figure 5.21). The figure shows cumulative losses. It is clear in this case that sorption plays a significant role. These trends are apparent for all the different primary chain lengths where losses occur, but the extent of loss decreases with increasing primary chain length and molecular mass. In addition, the number of loss mechanisms involved gradually decreases with increasing primary chain length. For methyl- isomers all mechanisms contribute to overall losses, whereas solely biotic, photo-oxidation/volatilisation losses occur for propyl- and pentyl- isomers.

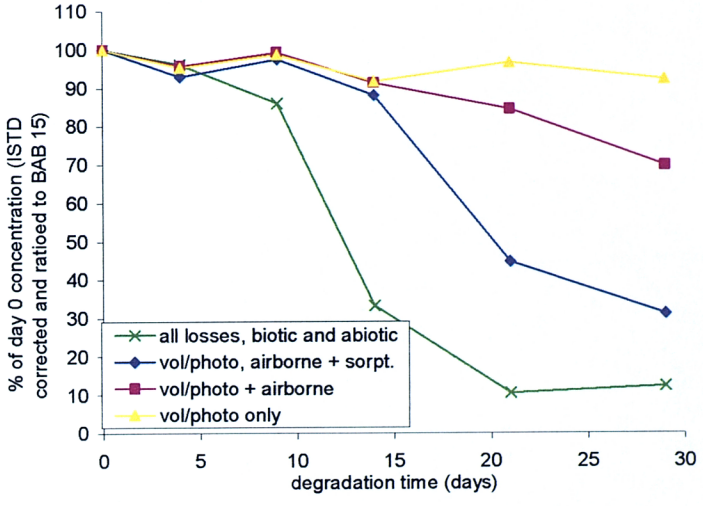


Figure 5.21 Cumulative contribution of the different loss types for the C_{18} methyl-isomer in ES. This data incorporates both an internal correction using the d_8-naphthalene peak, and ratioing to the C_{19} pentyl- isomer (BAB peak 15). The difference between the curves indicates the relative contribution of each of the mechanisms

The presence of other carbon sources, as in a bioreactor containing EcoSafe™ (ES), has an impact on the loss profile of BAB (Figure 5.22). The graph shows that there both rate and extent of compound loss are reduced with the presence of the other ES components acting as possible alternative carbon sources. The extent of this inhibition is most marked at higher molecular mass (C_{19}) and shorter primary chain lengths (methyl-). A 9-day lag phase is evident.

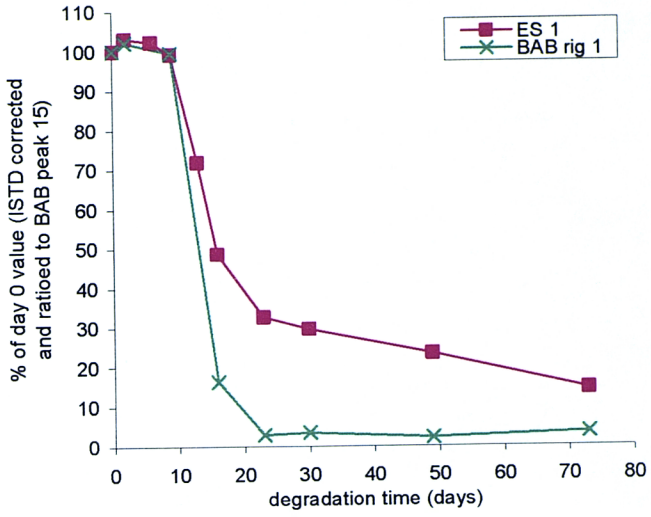


Figure 5.22 C_{18} ethyl- isomer losses with time in bioreactors with AS and different carbon source compositions. The BAB rig 1 contains BAB as the sole carbon source, whereas the ES 1 bioreactor contains EcoSafe™ in its entirety. This data incorporates both an internal correction using the d_8-naphthalene peak, and ratioing to the C_{19} pentyl- isomer (BAB peak 15)

Outcomes:

- **molecular mass and secondary chain length have a smaller impact on concentration changes than does the primary chain length,**
- **BAB losses occurring – yes, with increased losses for the shorter primary aliphatic chain lengths (methyl- and ethyl- isomers)**
- **Abiotic mechanisms involved – all types evident for the methyl- and ethyl- isomers, number of loss types involved decreases with increased primary chain length,**
- **Dominant loss mechanism – neither,**
- **Lag phase apparent – yes, generally lasting about 9 days, and**
- **Losses affected by the presence of other compounds – yes.**

5.5.5 Bis-methylsytryl benzene (bis-MSB)

This compound is a component of both Gold Star™ and EcoSafe™. Although it was detected by GC-MS, peak areas were too small to clearly distinguish it from the baseline and detect any significant changes, therefore, data for this compound are not available.

5.5.6 Loss of analytes as part of the liquid scintillant cocktails

Some liquid scintillant components are affected by the presence of other analytes that could potentially be used as alternative carbon sources (Sections 5.5.2 and 5.5.4). BEE showed large losses in GS and was not affected by the presence of other compounds. PPO showed slower losses in GS than in ES. As the major difference between the two cocktails is the primary solvent used, it is likely to be this that is the controlling factor. It would appear therefore that it is DIPN, and/or BEE, rather than the BAB that is causing most inhibition of PPO losses. DIPN showed no losses in either GS or when it was present as a sole carbon source and therefore no conclusions on the inhibitory effects of any other compounds in GS can be drawn from this data. BAB loss was slightly inhibited by the presence of other compounds in ES, and no data were available for bis-MSB or the emulsifiers (nonylphenol ethoxylates).

5.5.7 Rates of loss, half-lives and lag times

In order to compare the losses in different experiments, first order rate calculations were carried out to provide values for rate constants and half-lives. These calculations have been used by numerous studies for this purpose. Mohammed *et al.* (1996) stated that the removal of a variety of compounds under both laboratory and field experiments have conformed to first order kinetics. Quiroga *et al.* (1999) questioned this, but agreed that these rate equations could be applied to laboratory experiments. Kirby (1994), Stagg *et al.* (1996) and Susarla *et al.* (1998) also used first order rate equations to calculate pollutant half-lives for comparison purposes.

In this study, rate constants and half-lives were calculated using first order rate equations from Atkins (1988). All key degradation data was assessed by this means.

Ln $[A]_t/[A]_0$ was plotted against time (Figure 5.23), the slope determined using regression analysis giving a value for k, and the half-life was calculated using:

$$t_{1/2}\ (d) = \ln (2) / k$$

where: $[A]_t$ is the concentration of compound A at time t

$[A]_0$ is the concentration of compound A at t=0

$t_{1/2}$ is the half-life, given in days

ln (2) = 0.693, and

k = rate constant.

Slope was calculated from a regression line analysis of the region of the curve following the lag phase and prior to the stable concentration plateau (Figure 5.23). Exclusion of the plateau section for rate calculations was also carried out by Potter *et al.* (1999b), and was compensated for by Quiroga *et al.* (1999).

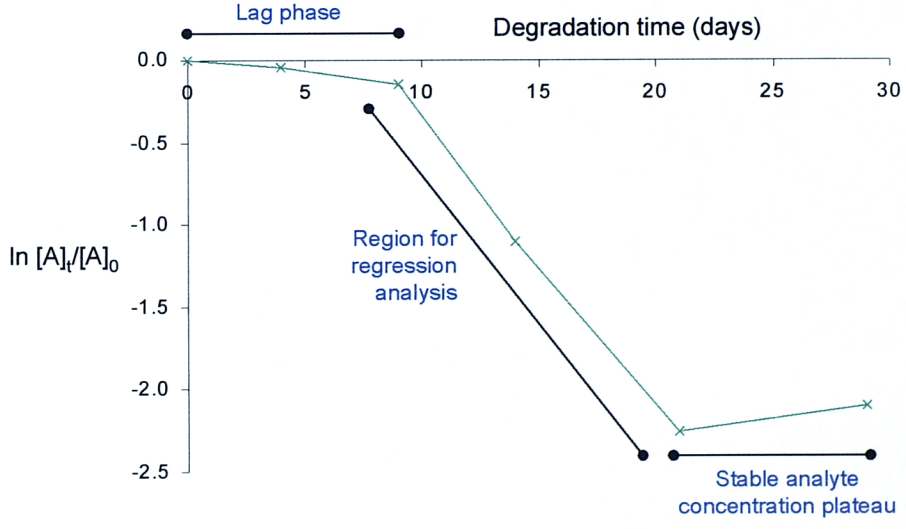


Figure 5.23 Typical profile for regression line analysis showing lag phase, area of regression analysis and plateau of stable concentration

Interpreted data are given in Tables 5.8 and 5.9, where rate constants have been used to calculate half-lives. Half-lives can only be used if calculated from the end of the lag phase, hence the duration of this initial phase is important.

Table 5.8 Relative half-lives (given in days) of certain key compounds. The time span of losses (days following the start of the biodegradation experiment) over which the regression analysis was calculated is given in brackets and blue type

Compound	*A+p+v (control bioreactor)*	*All losses*
C_{18} pentyl- in EcoSafe™	1730 (9-30)	410 (9-30)
C_{18} butyl- in EcoSafe™	No trend	160 (9-30)
C_{18} propyl- in EcoSafe™	1160 (9-30)	50 (9-30)
C_{18} ethyl- in EcoSafe™	3470 (9-30)	9 (9-30)
C_{18} methyl- in EcoSafe™	70 (9-30)	2 (9-16)
C_{16} ethyl- in EcoSafe™	50 (9-30)	5 (9-16)
C_{17} ethyl- in EcoSafe™	80 (9-30)	8 (9-30)
C_{19} ethyl- in EcoSafe™	230 (9-30)	10 (9-30)
PPO in EcoSafe™	1 (9-20)	1 (2-9)
PPO in Gold Star™	No data	1 (9-16)
BEE in Gold Star™	No data	1 (0-9)
DIPN 1 in Gold Star™	No data	80 (9-30)
DIPN 2 in Gold Star™	No data	120 (9-30)

Table 5.9 Relative half-lives of certain key compounds with details for different loss types

Compound	*P+v losses*	*A+p+v (control bioreactor)*	*S+a+p+v*	*All losses*
C_{18} pentyl- in EcoSafe™	No trend	No trend	No trend	No trend
C_{18} butyl- in EcoSafe™	290 (14-29)	410 (14-29)	530 (14-29)	130 (14-29)
C_{18} propyl- in EcoSafe™	870 (9-29)	390 (9-29)	690 (9-29)	80 (9-29)
C_{18} ethyl- in EcoSafe™	690 (9-29)	630 (9-29)	120 (9-29)	20 (9-29)
C_{18} methyl- in EcoSafe™	40 (9-29)	330 (9-29)	10 (9-29)	4 (9-21)
C_{16} ethyl- in EcoSafe™	140 (9-29)	110 (9-29)	80 (9-29)	10 (9-29)
C_{17} ethyl- in EcoSafe™	210 (9-29)	360 (9-29)	100 (9-29)	20 (9-29)
C_{19} ethyl- in EcoSafe™	2310 (4-29)	1160 (4-29)	290 (4-29)	20 (4-29)
PPO in EcoSafe™	1 (4-9)	0 (4-9)	1 (4-9)	0 (4-9)
PPO in Gold Star™	1 (4-9)	1 (4-9)	0 (4-9)	1 (9-14)
BEE in Gold Star™	1 (4-14)	0 (4-9)	0 (0-4)	0 (0-4)
DIPN 1 in Gold Star™	500 (14-29)	30 (14-29)	30 (14-29)	20 (0-21)
DIPN 2 in Gold Star™	300 (14-29)	30 (14-29)	30 (14-29)	20 (0-21)

Key to losses: P+v = photo-oxidation + volatilisation (other losses)
A+p+v = airborne contaminating microbes, photo-oxidation + volatilisation
S+a+p+v = sorption, airborne contaminating microbes, photo-oxidation + volatilisation (all abiotic)
All losses = biotic and all abiotic

Table 5.8 shows that when all losses are able to occur, compound half-life is reduced, e.g. from 230 to 10 days for the C_{19} ethyl- isomer in EcoSafe™. This trend is also generally borne out by the data given in Table 5.9. In addition, the time span over which losses occur (in blue), are both brought forward in time and reduced. A good example of this is PPO in Gold Star™, who's losses occur between days 9 and 20 in the control bioreactor and days 2 to 9 in the 'all losses' bioreactor. BEE in Gold Star™ in Table 5.9 also shows this pattern. It should be noted that a reduced compound half-life corresponds to an increased degradation rate.

In addition to trends being established with increasing number of loss mechanisms, BAB primary aliphatic chain length and molecular mass also affect half-lives. This data reconfirms the conclusions drawn in Section 5.5.4 regarding impact of BAB structure on degradation rates.

When looking at the C_{18} isomer data as presented in Table 5.9, it is evident that with an increase in primary aliphatic chain length (methyl- to pentyl-) there is a general increase in isomer half-life, corresponding to a decrease in degradation rate. When considering the ethyl- isomers with increasing molecular mass (C_{16} to C_{19}), again an increase in half-life is apparent. Despite this, C_{19} ethyl- isomer has only a 4-day lag time, in comparison to 9 or more days for the other BAB isomers.

These tables reiterate the trends mentioned in the sections above, showing that half-lives decrease and hence degradation rates increase with:

- increasing number of loss types,
- decreasing BAB aliphatic chain length, and
- decreasing BAB molecular mass.

BEE and PPO both show rapid losses, corresponding to very short half-lives. PPO degradation is marginally slower in GS than in ES (Table 5.8 and 5.9). DIPN is proving to be very recalcitrant, with long half-lives, particularly in the data shown for Batch A in Table 5.8, and the two isomers act in a very similar

fashion. As for the BAB data, this goes to re-iterate the conclusions drawn in Sections 5.5.1 to 5.5.3.

On considering the lag times, it is the compounds most resistant to losses that have the longest lag times, whereas those least resistant to losses, like BEE, have a very short lag phase. As shown by BEE in GS, when more loss mechanisms are occurring not only is the duration of the reaction period reduced, but the onset of losses is also brought forward in time.

5.5.8 Biodegradation products

The data show an absence of any degradation products in the GC-MS chromatogram, hence pathways cannot be elucidated.

5.5.9 Reproducibility of the experiment

In addition to the sampling and analysis variability discussed previously, experimental reproducibility was also assessed. Two batch experiments were carried out, at different times of the year and with a different starting biomass. The laboratory conditions, including nutrient mix composition, compound concentration and aeration rates, were standardised, but this could not include air temperature and incident sunlight.

Reproducibility data (Table 5.10) are given as percentage of the initial concentration remaining on day 30. In addition to the extent of loss, rate constants of the degradation process are also given (in brackets and blue type). Again all data and calculations for rate constants are for the period used for regression analysis. Data given in red type are those considered to be 'readily degradable' by the OECD definition. This involves 60% losses of the original concentration in 28 days (here figures are given to 30 days) and a maximum duration of 10 days between the end of the lag phase and the beginning of the stable concentration plateau (Staples *et al.*, 1999; Žgajnar Gotvajn & Zagorc Končan, 1999).

The table shows that even when rate constants do not show a good comparison between the two experiments, the percentage remaining is often highly

comparable. There are some exceptions however, such as DIPN in GS (all losses), C_{18} ethyl- isomer in ES (all losses) and C_{15} ethyl-isomer in ES (all losses).

Table 5.10 Comparison of the two biodegradation experiments (A and B) regarding extent and rate of loss. Figures in red represent loss scenarios where the compound can be classified as biodegradable according to the OECD definition. Figures in black are % of original concentration remaining at day 30, and figures in blue and brackets are for the rate constant of the degradation profile.

Compound	*ES all losses - **A***	*ES all losses - **B***	*ES a+p+v - **A***	*ES a+p+v – **B***	*GS all losses - **A***	*GS all losses - **B***
BEE	N/a	N/a	N/a	N/a	0 (1.08)	0 (2.30)
DIPN 1	N/a	N/a	N/a	N/a	100 (0.01)	70 (0.03)
DIPN 2	N/a	N/a	N/a	N/a	100 (0.01)	69 (0.03)
PPO	0 (0.96)	0 (1.53)	0 (0.60)	0 (1.62)	0 (0.81)	0 (1.45)
C_{18} pentyl-	99 (<0.01)	97 (<0.01)	96 (<0.01)	99 (<0.01)	N/a	N/a
C_{18} butyl-	91 (<0.01)	94 (0.01)	100 (<0.01)	97 (<0.01)	N/a	N/a
C_{18} propyl-	74 (0.02)	82 (0.01)	97 (<0.01)	95 (<0.01)	N/a	N/a
C_{18} ethyl-	30 (0.08)	52 (0.03)	92 (<0.01)	98 (<0.01)	N/a	N/a
C_{18} methyl-	6 (0.35)	12 (0.18)	77 (0.01)	92 (<0.01)	N/a	N/a
C_{16} ethyl-	12 (0.13)	26 (0.06)	46 (0.01)	85 (0.01)	N/a	N/a
C_{17} ethyl-	17 (0.09)	42 (0.04)	80 (0.01)	95 (<0.01)	N/a	N/a
C_{19} ethyl-	27 (0.07)	52 (0.03)	96 (<0.01)	100 (<0.01)	N/a	N/a

Key: N/a = not applicable
ES = EcoSafe™
GS = Gold Star™
All losses = all biotic and abiotic losses
a+p+v = all losses due to airborne contaminating microorganisms and photo-oxidation/volatilisation
A = batch experiment A
B = batch experiment B

5.6 Discussion and conclusions of the GC-MS data

This section will discuss each of the main biodegradation areas in turn, namely: lag phase, abiotic losses, biodegradation mechanisms, products and preferences, degradation rate, and reproducibility of the process. As these characteristics are often interdependent there is some overlap between the sections.

5.6.1 Lag phase

The literature review given in Section 2.6.1 suggested that the use of a mixed microbial culture should result in a reduced lag time, which was anticipated to be several days depending on the presence of other carbon sources. In addition, lag times correlating with the suggested order of preferential metabolism were expected for compounds in the liquid scintillant cocktail mixtures.

Lag phases of compounds varied from 0 days for BEE as a sole carbon source, to 14 days for DIPN and C_{18} butyl- BAB isomer in liquid scintillant cocktails. These last two compounds are also those considered to be the most recalcitrant. In addition, the increased resistance to biodegradation of the longer primary chained BAB isomers corresponded with increased lag times. Generally the longer lag times were evident for the compounds most resistant to biodegradation. This correlation between reduced biodegradability and extended lag times suggests that the biomass takes a long time to adapt to metabolising these recalcitrant compounds, and even when adaptation does occur metabolism is only very minor. As found for this study, Susarla *et al.* (1998) stated that analyte type played an important role in compound lag time.

In addition to biodegradability, the presence of other carbon sources also played a role in influencing lag times. PPO lag time was increased when it was present with other compounds in Gold Star™ and EcoSafe™. This suggests that there may be preferential metabolism of other compounds in the liquid scintillant cocktails that is resulting in the delayed onset of compound losses. As the 'first choice' carbon source is exhausted there is a shift to the metabolism of the less available compounds, this could either be by the same microorganisms or alternatively a shift in population structure occurs with the adaptation of specific microorganisms suited to the 'second choice' carbon source.

In addition to analyte type and presence of other compounds, the loss mechanisms involved also influenced lag time. Lag times were found to be longer when solely abiotic losses were occurring in comparison to additional biotic losses with the use of a viable biomass. This is shown by the PPO in

EcoSafe™ data in Table 5.8, where lag times were 9 days in the control bioreactor, with only some abiotic losses occurring, and just 2 days when all losses (abiotic and biotic losses) occurred. This suggests that the inclusion of biotic losses reduces the lag time. This is likely to be a direct consequence of an additional loss mechanism occurring.

5.6.2 Abiotic losses

Large abiotic losses were found in some cases, including up to 100 % in the case of PPO and 54 % for the C_{16} ethyl- BAB isomer. Four main categories of loss mechanism were investigated, namely: volatilisation/photo-oxidation, losses as a result of airborne contaminating microbes, sorption, and biotic losses. Sorption was considered a true loss rather than a reversible compound sink, as it results not only in the compound being unavailable for mineralisation by the biomass, but also unavailable for extraction and analysis. This is the reasoning used by Ressler *et al.* (1999) in assessing sorptive losses for polyaromatic hydrocarbons.

Practical differentiation between the loss types is elaborated on in Section 3.4.2. It should be noted that losses by these mechanisms were not measured directly, but rather inferred from cumulative data as a result of experimental set-up. The data shows a good comparison between expected and actual losses (Table 5.11). Expected losses are those stated in Section 2.6.2 for related compounds.

Extensive losses for BEE, both abiotic and biotic, were expected and indeed occurred. The low molecular mass of this compound makes it a prime candidate for volatility losses. The sorptive properties make BEE comparable to nonylphenol ethoxylates, suggesting that it is the ethoxylate and ethoxylate-like structures that contribute to this property.

Despite the substantial expected DIPN losses, these were not apparent in this study, with only very minimal sorptive losses occurring. The statement by Addison *et al.* (1983, as cited in Addison, 1983) regarding the volatility and sorption of DIPN does not give any indication of the time span involved, and

therefore it may be such that these losses will occur to a greater extent given a longer experimental duration. DIPN losses are only seen when the compound was present as part of Gold Star™, and not when used as the sole carbon source. The reason for this is uncertain and could not have been as a result of co-metabolism as DIPN losses are purely abiotic.

Table 5.11 Expected and actual loss mechanisms of the different compounds of interest. Expected losses are those given by the literature for related compounds.

Compound	*Expected losses*	*Actual losses*	*Notes*
BEE	- volatilisation (+)	- volatilisation and photo-oxidation (+) - sorption (+)	
DIPN	- sorption (+) - volatilisation (+)	- no substantial losses	
PPO	- sorption (-)	- volatilisation and photo-oxidation (+) - sorption (-)	
BAB	- sorption (+) - volatilisation (-)	- volatilisation and photo-oxidation (+) - sorption (+)	Extent of loss is a function of both primary chain length and molecular mass
Bis-MSB	- sorption (-)	- unknown	not detected

Key: (+) = large losses
(-) = small losses

Small losses of PPO through sorption were apparent, along with larger photo-oxidative/volatilisation losses. Little is given in the literature regarding abiotic losses of this compound, although the sorption properties had been predicted on the basis of PAH sorption. The additional abiotic losses contribute to the rapid loss of PPO with time.

Linear alkyl benzene data were used to predict BAB losses. There are very few references regarding branched alkyl benzenes, most referring to them as alkyl benzenes. This however does not differentiate on the basis of chain length, a factor found to be so important in this study. All abiotic mechanisms, particularly photo-oxidation/volatilisation and sorption, contributed to the losses found, but these varied markedly depending on isomer structure. Both the extent of loss

and the number of loss types decrease with increasing primary chain length and molecular mass. The data show that on increasing primary aliphatic chain length from C_1 to C_5, within a specific molecular mass group, loss mechanisms change from all loss types to solely volatilisation/photo-oxidation. It is not understood what is contributing to this phenomenon, however, the correlation with the ease of metabolism is evident, and possible explanations are outlined in Section 5.6.3.

A further query is with the data for the bioreactor with photo-oxidation/ volatilisation losses and losses due to airborne microbial contamination (p+v+a). These results sometimes show artificially high or low half-lives (Table 5.9). There appears to be a correlation between the highly recalcitrant compounds and unexpectedly low half-lives as for the BAB isomer C_{18} butyl- and DIPN, and *vice versa* for the C_{17} ethyl- isomer. Low half-lives reflect high rates of removal. Thus, artificially high loss rates are evident for the highly recalcitrant compounds in the a+p+v bioreactors. This can only be as a result of the addition of a bactericide to the p+v bioreactor, as this is the sole difference between the p+v and a+p+v bioreactors, however, an explanation cannot be found, particularly regarding the variable effect with compound structure and recalcitrance. It is most likely that the a+p+v bioreactor is artificially decreased or increased, as it is this one that repeatedly falls out of the trend established by the other three bioreactors.

5.6.3 Biodegradation mechanisms and products, preferences and rates

5.6.3.1 Mechanisms and products

Despite use of the GC-MS in full scan mode to enable the detection of any degradation products, none was detected.

It might have been expected to be able to detect a progressive loss of certain compounds and the generation of any intermediates, which in turn decreased in concentration as they were metabolised. Lack of this detail suggests that this intermediary stage is not occurring, the metabolites are not being identified, or that the sampling frequency of the experiment precluded any of these small timescale changes from being detected. It seems unlikely that this sequential

metabolism did not take place, thus the intermediates are undetected either due to the sampling frequency or an analytical problem.

With the mixed culture used, it was hoped that biodegradation would not be inhibited at any stage of the pathway, and this indeed appears to have been the case as no metabolic intermediates have been detected. On the basis of this explanation, the data would suggest that a programme of more frequent sampling would detect sequential compound biodegradation. Without any information regarding the metabolic intermediates it is also impossible to determine the degradation pathways involved.

Assuming that the degradation rate can be detected within the present sampling regime, one would expect to detect intermediate metabolites of the reactions. Possible intermediaries would include phenols and toluene. If considering BAB degradation it seems possible that initial microbial attack would occur on the primary aliphatic chain, producing a linear alkyl benzene with a free radical at the point at which the primary chain was attached. The activity of free radicals could have resulted in the combination of several of these products. The large molecular mass would then mean that they were no longer within the dynamic detection range of the GC-MS apparatus, and therefore could only be detected by another mass spectrometric detector such as a Time-of-Flight (ToF) or Fourier Transform (FT) mass analyser.

The lack of detected biodegradation end products is also puzzling. It was anticipated that alcohols would be the principle products. Due to the small molecular weights of some of the alcohols, these products are unlikely to be detected by GC-MS analysis as they would pass through the system within the solvent front or the programmed solvent delay prior to the start of detection. In addition, any fatty acids produced are most likely to be trapped in the injector or at the head of the column (Langley, 2001, *pers comm.*). Some of the degradation products may also have been lost through volatilisation. These could possibly be detected by the use of a closed system with a trap for the effluent gas. A further possibility is that both the smaller intermediates and

products irreversibly sorbed onto the biomass, thus removing them from solution and preventing their detection.

5.6.3.2 Biodegradability and preference

Despite the lack of results regarding degradation pathways and products, it was evident from the data that biodegradation (biotic losses) of certain key compounds did occur in addition to any abiotic losses. BEE, PPO and some of the BAB isomers are shown to be biodegradable under the conditions used. These conditions were very simple laboratory conditions including; a basic nutrient medium, simple aeration system, open bioreactor, no temperature or pH control and the use of activated sludge (AS) as a microbial source. DIPN and BAB isomers with 3 - 5 carbon atoms in the primary aliphatic chain were shown not to be readily biodegradable. DIPN does not show metabolic losses even when co-present with other carbon sources, as is the case in Gold Star™.

The reason for the trend of decreasing biodegradability with increasing primary chain length is not well understood, and nothing was found in the literature to suggest an explanation. However it is clear from the data that preferential metabolism occurs on the shorter primary aliphatic chains, despite a longer secondary chain and the same number of carbons overall. As to why there is only a trend with primary chain length, and not with secondary chain length, is also uncertain.

To attempt an explanation for the preferential biodegradation of the shorter alkyl chains, trends for degradation orders from other studies have been used. Alkane biodegradation is highest for short chain lengths (Hakstege & van Geldermalsen, 1998; Prince, 1993; Venosa *et al.,* 1993) and branching will have reduced the BAB' susceptibility to biodegradation (Britton, 1984; Ejlertsson *et al.*, 1999) in comparison to simple alkyl benzenes. Short primary chain BAB biodegradation should result in intermediaries that have a single long secondary chain lengths. Although the longer chain is less susceptible to microbial attack than any remaining short alkyl chains, the intermediate compound will be a simple alkylbenzene. The lack of branching will result in this being more easily

biodegraded than any branched alkyl benzenes, and will thus ensure its continued metabolism.

On the basis of this hypothesis, sequential biodegradation of the shortest to the longest primary chain lengths would occur, with intermediate metabolites being used as a carbon source prior to microbial attack on the next BAB in the sequence. As no relevant literature could be found, this suggested sequence of events does not take into account any effect from the presence of a benzene ring.

Focht (1988, as cited by Lindow *et al.*, 1989) stated that even if individual compounds are biodegradable, mixtures sometimes are not. Although there may have been some inhibition of the onset of mineralisation of some compounds in the liquid scintillant cocktail mixtures, there was never a case of compounds being classed as biodegradable when present as a sole carbon source and recalcitrant when in combination with other compounds. It was primarily PPO that showed biodegradation inhibition when present in liquid scintillant cocktails. This may have been as a result of preferential metabolism of another carbon source. The most likely alternative carbon sources are BEE or the methyl- and ethyl- BAB isomers.

Analytes that can be classed as 'readily biodegradable' according to the OECD classification (shown in red) include BEE, PPO and the C_{18} methyl- and C_{16} ethyl- BAB isomers for batch A only (Table 5.10). Although extensive losses did occur in batch B, the duration of loss prior to the start of the stable concentration plateau exceeded the statutory 10 day period. It is these same analytes, and a few more BAB isomers besides, that this study has classed as readily biodegradable. Therefore the OECD definition which applies to tests conducted by oxygen consumption and carbon dioxide emission measurements, can also be used for analyte concentration monitoring biodegradation tests as for this study.

The result for DIPN recalcitrance was most unexpected as a study by Severn Trent Laboratories in 1989, on the biodegradability of Optiphase Hi-Safe™,

regarded it as 'readily biodegradable'. The primary solvent in this liquid scintillant cocktail is DIPN. The biodegradation test was carried out at a Optiphase Hi-Safe™ loading of 0.005 % and using activated sludge (AS) as the microbial source. This study indicates that losses can occur at very low concentrations. It must be noted however that no actual compound concentration analyses were conducted and that biodegradation was inferred from daily dissolved organic carbon (DOC) measurements over a four-day period. The use of this technique results in doubt as to whether the liquid scintillant cocktail components themselves are being metabolised or whether it is solely a decrease in sludge carbon levels. This is particularly true as changes in DOC for a control sample of AS were not determined, and this biomass source will certainly have contained some carbon source when it was obtained. The results from the present study suggest that the ISO 7827 method used in the Severn Trent Study may limit the interpretation regarding the true biodegradability of a test compound.

The present study was conducted at a liquid scintillant cocktail (LS cocktail) loading of 1 %, or the equivalent concentration of DIPN. These concentrations are much closer to, but still less than the levels that would be required for a commercial biodegradation process to be considered viable. At this concentration and under the experimental conditions used in this study, DIPN cannot be considered to be readily biodegradable (Figure 5.24). This poses a problem both for the LS cocktail manufacturers that label their DIPN-based LS cocktails as biodegradable, and for the use of biodegradation as an alternative means of waste disposal. The same applies to LS cocktails that contain the range of BAB isomers shown in EcoSafe™. However, several of the BAB isomers are biodegradable at a 1 % loading level (Figure 5.24), and therefore if there is selective use of these isomers, the cocktails can indeed be classed as biodegradable, and commercial biodegradation of these chemical mixtures can be carried out.

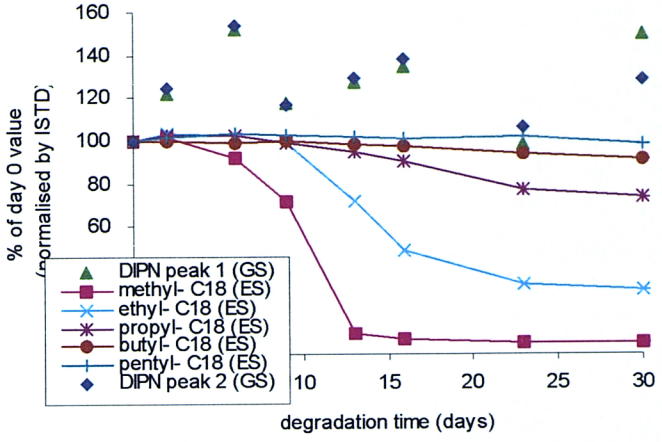


Figure 5.24 Total losses (abiotic and biotic) for the two primary solvents dealt with in this study. Note that fluctuations in the DIPN data are not attributable to concentration change, but rather to the absence of a correction factor for bioreactor heterogeneity. The BAB data does include a correction for this purpose and therefore the fluctuations are absent. Details in brackets indicate the liquid scintillant cocktail in which the solvent is present

On the basis of the literature and the constituent groups of the compounds of interest, the expected sequence for degradation order was:

- BEE (ethoxylate and aliphatic),
- BAB (branched aliphatic and 1-ring aromatic),
- PPO (oxazole and 1-ring aromatic),
- Bis-MSB (unsaturated aliphatic and alkyl benzene), and
- DIPN (branched aliphatic and 2-ring aromatic).

The data in this study confirmed this sequence, with BEE showing instantaneous losses both as a sole carbon source and when present in GS. A 9-day lag time for BAB losses occurs both with (BAB 1 bioreactor) and without the presence of other analytes (ES bioreactor), therefore it is metabolised after BEE, but in preference to other compounds with a longer lag time. PPO only shows inhibition in GS, probably as a result of the co-presence of DIPN, and DIPN shows no substantial losses at all, whether alone or with other compounds. Data for bis-MSB was unobtainable and therefore no conclusions can be drawn for this component.

5.6.3.3 Rates of loss

These vary markedly as a function of compound structure. Largest overall losses correlate with highest loss rates, and hence also degree of biodegradability (Table 5.10). Biodegradation rates also have an impact on the time span between the end of the lag phase and the end of maximum degradation (the start of the stable concentration plateau), with higher rates resulting in a reduced time span, as in the case of PPO in EcoSafe™ (Table 5.8). The first order rate equations used to calculate rate constants and half-lives provide data which is comparable to first estimates of these parameters, suggesting that the correct equations were used.

5.6.4 Reproducibility

5.6.4.1 Reproducibility of the process

Potter *et al.* (1999b) conducted a study into removal of nonylphenol ethoxylates in wastewater treatment plants in the summer and winter. They considered removal rates in duplicate experiments of 45 and 68 %, and 64 and 74 % to show lack of system variability. The results from this study are on the whole comparable to these reproducibilities.

Considering the large variations in operation conditions used for the two experiments of this study, the degradation process can be considered to show reproducibility. Despite a slight variation in rate constants of the two batches, the extent of removal is generally comparable in a given time frame. This is important when using the biodegradation technique in a commercial set up as it rules out the requirement for stricter controls on operating conditions and microbial stock used for the procedure.

5.6.4.2 Reproducibility of the data

There are two main types of error, systematic and random (Howard & Statham, 1993). Systematic errors involve constant, fixed absolute or relative error and affect the accuracy of a measurement. A possible source of systematic errors is extraction efficiency variations, but these can be easily corrected for by a single factor multiplication. Random errors are more difficult to correct for as they can be a factor of storage or analysis problems, batch failures or rogue results. It is

with these errors in mind that replicate analyses are carried out. Within the procedure carried out for this study the most likely sources of error are storage, sample preparation (homogenisation and sub-sampling), liquid-liquid extraction, and injection.

The use of BAB ratioing to the BAB 15 isomer (C_{19} pentyl- isomer) greatly improved the data by limiting the variability as a result of non-representative sampling. No such correction was available for the PPO, BEE and DIPN data thus making this much more variable, as is clearly shown by oscillations in the data. As mentioned previously, this technique of using an internally conserved standard to correct for sampling errors, mixing problems etc. has been extensively used elsewhere. Despite its use being questioned, these corrections provide reasonably accurate results in short-term assays (Santas *et al*, 1999). Variability of the PPO, BEE and DIPN data may have been reduced had an artificial internally conserved standard such as hopane, pristane or phytane been added at the start of each experiment and used for correction purposes.

Despite time and monetary limitations restricting the number of replicate experiments, samples, extractions and analyses that could be carried out, the data shows good reproducibility (Table 5.12). All these stages fall within acceptable error limits, and result in a cumulative error of 8.6 % using the 'sum of squares' method.

Table 5.12 Relative standard deviations of the different stages of GC-MS analysis. The data given is average relative standard deviations from three separate experiments in each case

Stage of GC-MS work	*Relative standard deviation (%)*
Sampling and liquid-liquid extraction	6.5
Injection and analysis	5.5
Peak choice by retention time	0.1
Manual peak area integration	1.0

Several factors may have combined to increase the error of the technique, including:

- only a few samples being analysed in triplicate. This was primarily as a result of time restraints and could be improved by routine triplicate sample analysis,
- the use of a single syringe for sampling. Despite it being rinsed several times between each sample being taken, some cross-contamination may have occurred. Therefore any future work could use a separate syringe for each bioreactor,
- extensive storage periods between sampling and analysis on some occasions, which may have affected sample composition. This duration will have to be minimised or a thorough assessment of storage and freezing-defrosting cycle losses of the analytes will need to be conducted. Included in this study would be storage aspects such as whether or not filtration prior to freezer storage would be advantageous.

5.6.5 Conclusions

The GC-MS work was instrumental in answering several of the key questions regarding the biodegradation of the DIPN and BAB based liquid scintillant cocktails (Gold Star™ and EcoSafe™), although several aspects remain unanswered. It was evident that a lag phase occurred for most compounds prior to the onset of any losses. Lag times varied markedly, but were primarily controlled by compound structure, the co-presence of other compounds, and the number of loss mechanisms involved. Due to a lack of data regarding products and intermediary metabolites of the biodegradation, pathways could not be determined.

Both abiotic and biotic losses occurred for the majority of compounds. They were clearly a function of compound structure, with high losses being found for PPO, BEE and BAB isomers with one or two carbons in the primary alkane chain. Low abiotic losses were evident for DIPN and the remaining BAB isomers. The extent of loss correlated with the overall recalcitrance of the compound.

The loss profiles were shown to conform to first order rate kinetic equations. However, in order to calculate rate constants and half-lives using these equations both the lag phase and the stable concentration plateau following the time of maximum losses had to be excluded. As well as abiotic loss extent, rate of loss was also found to correlate with overall recalcitrance. Loss rates had an impact on loss duration, with higher rates relating to shortest loss periods.

Despite slight variations in rates of loss, the extent of loss was comparable between the two experiments. Therefore despite the variable operational conditions and biomass source used, the process can still be considered to be reproducible.

Regarding the use of biodegradation as an alternative waste disposal mechanism, under the experimental conditions and concentration levels used, BEE, PPO and BAB isomers with one or two carbons in their primary aliphatic chain can be regarded as 'readily biodegradable'. However, DIPN and BAB isomers with three to five carbon atoms in their primary aliphatic chain cannot. This poses a potential problem for liquid scintillant cocktail manufacturers using BAB and DIPN as their primary solvents, as these cocktails should not be labelled 'biodegradable' unless selective BAB isomers only are used.

Chapter 6 - Discussion and process development for the biodegradation of liquid scintillant cocktails

This chapter combines the outcomes highlighted in Chapters 4 and 5 regarding the microbial and GC-MS characterisation of liquid scintillant cocktail biodegradation, and discusses future work on the basis of these results.

The aims of the project included:

1. assessing the biodegradation of liquid scintillant cocktails,
2. analysing and identifying degradation products,
3. determining optimum conditions for degradation using activated sludge, and
4. consideration of the process scale up issues, accompanied by a commercial viability study.

The work discussed in the previous chapters deals principally with aims 1 and 2 (above), with investigations being conducted on a laboratory scale only. For the programme to progress, an assessment of the optimised conditions to ensure maximum liquid scintillant cocktail biodegradation is necessary. In addition, in order to use biodegradation as a viable alternative to other waste disposal mechanisms, scale up, environmental impact assessment and commercial viability issues need to be addressed. Conclusions, and implications of this study are given in Chapter 7.

6.1 Are liquid scintillant cocktails biodegradable?

6.1.1 Correlation between microbiological and GC-MS characterisation

The techniques used in this study to answer this basic question were:

- microbiology - to determine biomass changes in relation to the presence of liquid scintillant cocktails and their constituents, and
- GC-MS analysis - to determine compound concentration variations with time in the presence of a viable microbial source.

A link between microbial growth and concentration losses is apparent (Table 6.1). Despite evidence of metabolism of certain compounds, this does not automatically indicate that a mixture of these same compounds will also be biodegradable (Focht, 1988, as cited in Lindow *et al.*, 1989).

Table 6.1 Conclusions drawn from the microbiological and GC-MS analytical work conducted in this study. Toxicity refers to effect upon activated sludge

Liquid scintillant cocktail or individual compound	*Microbiological outcomes (based on biomass growth)*	*GC-MS analytical outcomes (based on concentration change)*	*Combined outcomes*
Gold Star™ (GS)	Non toxic, available as a carbon source	Some components utilised by biomass	Non-toxic, available and some fractions used for growth
EcoSafe™ (ES)	Non toxic, available as a carbon source	Some components utilised by biomass	Non-toxic, available and some fractions used for growth
Butoxyethoxy ethanol (BEE)	Inconclusive	Utilised by biomass	Non-toxic, available and used for growth
Di-isopropyl naphthalene (DIPN)	Non toxic, not available as a carbon source	Not utilised by biomass	Non toxic, not available
Diphenyl oxazole (PPO)	Non toxic, not available as a carbon source	Large abiotic losses, utilised by biomass	Non toxic, available, large abiotic losses
Bis-methylstyryl benzene (bis-MSB)	Non toxic, not available as a carbon source	Unknown	Non toxic, not available
Branched alkyl benzenes (BAB)	Non toxic, available as a carbon source	Selective utilisation of shorter primary chain lengths	Non-toxic, available and some fractions used for growth

Conclusions drawn in Chapters 4 and 5 indicate that any decrease in concentration can, at least in part, be attributed to biomass growth and metabolism as a result of non-toxicity and availability of the carbon source. As the compounds most resistant to biodegradation were also those with the lowest biodegradation rates and biomass concentrations, it is true to say that biodegradation rates are proportional to total biomass. This observation was also made by Jobson *et al.* (1974) and Baughman *et al.* (1980, as cited in Spain & van Veld, 1983).

Biotic losses found by GC-MS analysis for BEE suggest that the compound is non-toxic, available and utilised for biomass growth. This correlates with agar plating experimental data, but not the optical density measurements (Table 4.7).

DIPN is shown to be non-toxic yet unavailable for use as a microbial carbon source, with no change in concentration being evident. Complete losses of PPO do occur which can be primarily attributed to abiotic losses.

Bis-MSB analysis was not carried out by GC-MS and therefore a comparison of this and the microbial technique cannot be made. However, it is unlikely that any biotic losses would occur, given that the microbial work indicated it to be non-toxic but unavailable as a carbon source. It must be noted however that although the biodegradation of bis-MSB has not been assessed, it is no longer a strictly necessary component of liquid scintillant cocktails. Modern photomultiplier tubes, as used in new liquid scintillant counters largely discount the need for this secondary fluor as they are highly efficient at the wavelengths of the primary scintillators (Kobayashi & Maudsley, 1974), including PPO.

As primary solvents are the principle components of liquid scintillant cocktails, it would seem unlikely that Gold Star™ as a whole, containing high levels of DIPN, would be considered an available carbon source. EcoSafe™ is more likely to be classed in this manner due to the selective BAB biodegradation of the shorter primary aliphatic chain lengths. DIPN may be resistant to biodegradation, but it has also been shown to be non-toxic, with both the microbiological tests and BOD_5 measurements showing a viable biomass present in bioreactors containing up to 40 % GS.

The use of surfactants to aid biodegradation of hydrophobic and non-aqueous phase liquid contaminants is well documented (Al-Hadhrami *et al.*, 1996; Choori *et al.*, 1998; Yeh *et al.*, 1998; Zhang *et al.*, 1999; Zimmerman *et al.*, 1999). The biodegradation process in this study may have been enhanced by the presence of nonylphenol ethoxylate surfactants, resulting in an increase in compound solubility and accessibility of the compounds to microorganisms, nutrients etc. However, even surfactants from the same homologous series can exhibit varying biological effects (Rouse *et al.*, 1994). As detailed tests have not been conducted the actual effects of this surfactant on the microbial biomass is unknown.

6.1.2 Additional conclusions and unresolved issues

Microbiological work indicated that neither plate counts nor optical density measurements could be used for truly quantitative analysis. In the case of agar plating this may have been a result of dilution, subsampling or counting errors. Furthermore, it proved that activated sludge could indeed be used as an effective microbial source for biodegrading some components of LS cocktails. GC-MS data provided further details of loss profiles and abiotic losses, showing that highest loss rates and shortest lag times occurred for the least recalcitrant compounds, e.g. PPO and BEE.

The study by Klein and Gershey (1990) into the biodegradability of liquid scintillant cocktails showed similar outcomes to this study, with biodegradation varying with cocktail primary solvent type. Linear alkyl benzene based cocktails were shown to be less resistant to biodegradation than those containing DIPN. The experiment used a liquid scintillant cocktail concentration of 0.01 %, activated sludge as the microbial source, and room temperature for a 7-day period. In their study no detailed assessment of abiotic losses was carried out. Xylene and trimethyl benzene based cocktails were also assessed but these showed extensive volatilisation losses and were therefore not compared with the other primary solvents. As for the present study, Klein & Gershey (*ibid.*) concluded that the recalcitrance of DIPN makes its classification as biodegradable questionable.

Neither technique was successful in identifying biodegradation products or intermediary metabolites and thus biodegradation pathways could not be determined. However, as mentioned in Chapter 5 (Section 5.6.3.1), if the degradation products are gaseous then a closed purged bioreactor system could be utilised. This would involve bubbling air through the system, acting as a carrier gas for any gaseous products, which could be trapped by a catalytic converter for periodic analysis. Alternatively, continuous monitoring of gas composition could be carried out using a specialised GC capable of detecting and quantifying organic compounds in a gaseous form. This second technique was also used by Lapham and co-workers (1999) for carbon dioxide quantification.

The trapping of effluent gases could also be used to give an indication of metabolic rates of specific labelled compounds. If BEE were to be radiolabelled with ^{14}C or ^{13}C, liquid scintillant counting could be used to monitor labelled carbon dioxide produced by mineralisation of this compound. This technique has been used in numerous biodegradation studies as an alternative to direct compound concentration monitoring by GC-MS or a comparable analytical technique (Al-Hadhrami *et al.*, 1996; Burgos *et al.*, 1999; Richnow *et al.*, 1998; Yoshida & Kojima, 1978b).

6.2 Planning process scale-up

In order for the process to be used in a commercial capacity, more biodegradation experiments will need to be conducted to further determine reproducibility. Repeated biodegradation experiments would also allow for the inclusion of an internally conserved standard such as hopane, to be used as a correction factor for DIPN, BEE and PPO, as the C_{18} pentyl- BAB isomer was used for BAB normalisation.

Following these repeat experiments, the laboratory set up would need to be scaled up to enable the processing of larger volumes of liquid scintillant cocktail waste. It should be noted however, that laboratory biodegradation rates may not be accurate predictors of rates found when the process is conducted on an increased scale (Floodgate, 1984; Staples *et al.*, 1999; Vikelsøe & Johansen, 2000). However, models such as that developed by Okey & Stensel (1996) to predict biodegradation rates in activated sludge systems could be used for a first estimation.

6.2.1 Optimisation of controlling parameters

Prior to any scaling up process, factors most important in controlling the rate and extent of liquid scintillant cocktail biodegradation will need to be both determined and optimised, as they will influence the design of the scaled-up pilot plant and help predict the timeframe required for biodegradation to meet specific cleanup standards. Chapter 3 outlines some of the factors influencing biodegradation, including microbial source, nutrient medium composition,

operating temperature, oxygen availability and aeration, pH, and mixing. Control of any of these parameters will result in increased processing costs, thus reducing profit margins.

Table 6.2 Parameters to be tested for maximum biomass growth reflecting maximum liquid scintillant cocktail biodegradation, and effect on operational costs

Controlled parameter	*Variables to be tested for maximum biomass growth*	*Effect on process costs in comparison to current set up*
Temperature	Culture the growth media at ambient temperature, 30, 35 and 40 °C	Marked increase
Nutrient medium composition	Use of different standard nutrient media, including some with more trace elements	No marked effect
Nutrient introduction frequency	Use of nutrient additions after 1, 5, 10, 15 and 20 days, and numerous additions over a 30 day period	Slight increase
Liquid scintillant cocktail loading	Use of LS coctail concentrations at 1, 5, 10, 15, 20 and 30 %	Marked decrease
Biomass loading	Use biomass concentrations of 25, 50 and 75 %	Slight increase
Biomass source	Use of biomass adapted to LS cocktail at a 1 % level for 7, 14, 21 and 28 days	Slight increase

N.B. present activated sludge loading is 50 %

The maximisation of biodegradation involves an increase in loss rates and a reduction in lag times. Due to time limitations these could not be investigated during this study. On the assumption that many different controlling parameters will need to be assessed, a monitoring technique is required that provides results both readily and cost effectively. Outcomes from Chapters 4 and 5 showing compound concentration decreases mirrored by biomass growth would therefore suggest that population dynamics by means of optical density measurements, rather than the more time consuming and expensive use of GC-MS, may be best suited to this purpose. However, this technique would require refining for it to provide more quantitative data and hence be most useful. Due to the equipment set-up used for the optical density experiments, an investigation into the effect of variable aeration and mixing could not be carried out by this testing mechanism. The different parameters and recommended variables to be tested for biodegradation optimisation are given in Table 6.2.

Optimising these parameters will invariably reduce overall processing costs per unit volume of liquid scintillant cocktail waste.

6.2.1.1 Liquid scintillant cocktail concentration

Carbon source concentration is an important factor in this biodegradation process. A sufficiently high concentration is required to ensure maximum waste processing within a specified time period. However, concentrations should not be so high as to cause a toxic effect on the biomass (Leahy & Colwell, 1990). Data from the current study indicates that toxicity did not occur at a liquid scintillant cocktail concentration of 40 %. If these loading levels could be utilised for the commercial biodegradation process then large volumes of waste could be processed at any one time, markedly reducing overall costs. Klein & Gershey (1990) concluded that biodegradation was inversely proportional to initial cocktail concentration, and therefore used a liquid scintillant cocktail concentration of only 0.01 %. It is therefore important to further the preliminary investigations conducted in the present study.

6.2.1.2 Biomass adaptation

Adapting the biomass will take time and therefore will reduce the period for which any bioreactor can be operational. Adaptation of the mixed culture will result in an increase in both the proportion and numbers of microbes capable of metabolising the available carbon sources at the beginning of the biodegradation experiment (Floodgate, 1984). It must also be noted that any biomass adaptation must be carried out just prior to use, as there is a finite amount of time for which genetic capability resultant from the adaptation to a specific carbon source is retained within a population (Spain and van Veld, 1983). Adaptation was shown be to be effective tool to reduce lag times and prepare the biomass for elevated LS cocktail concentrations.

6.2.1.3 Temperature control

Artificially maintaining a bioreactor temperature above ambient room temperature will greatly increase running costs. Any increased process costs may be offset by enhanced biodegradation rates contributing to larger volumes

being processed within a given time period. If this control is considered to be necessary, insulation of the bioreactors will help to minimise heating costs.

6.2.2 Bioreactor type

Scaling up of the process should be based on the outcomes of the above mentioned investigations, and will require major modifications to the basic batch reactor set up used in this study. It is envisaged that a batch reactor will also be required for any scaled-up process, as this enables reuse of the biomass, sufficient retention time of the LS cocktail waste to ensure biodegradation, and control of process conditions and effluents.

Sequencing batch reactors (SBRs) are fill and draw activated sludge systems, where aeration and separation are carried out sequentially in a single tank (Tchobanoglous & Burton, 1991). As there is no loss of biomass there is no requirement for a return activated sludge system, with biomass being reused many times before replacement. Modifications include the use of oxygen and temperatures at 25 – 50 °C above ambient (Tchobanoglous & Burton, 1991), increasing both degradation rate and operating costs.

Furumai *et al.* (1999) used a SBR with a 100 L working volume, and automated controls to instigate each of the batch stages. With each cycle 25 L of the degradation supernatant was removed and replaced with fresh waste water. The biomass was used for a total of 80 cycles prior to being renewed. Glass & Silverstein (1998) also used SBRs due to their efficient reuse of biomass and ease of scale up.

6.2.3 Scale up of the liquid scintillant cocktail biodegradation process

Process parameters such as required retention time in the bioreactor and pump out time will need to be established. An experiment to determine the useful life span of the activated sludge in the bioreactors is also necessary.

In the laboratory experiments concentration losses of greater than 70 % are achieved in 30 days for BEE, PPO and the shorter primary alkyl chain lengthed BAB with non-adapted activated sludge. With improvement of conditions and

the use of pre-adapted sludge these biodegradation levels would hope to be achieved in 21 days. A model has been developed for a sequencing batch reactor on a 25-day cycle with no temperature or pH controls (Figure 6.1). This cycle period is composed of filling (6 hours), reacting (21 days), separating (1.5 days) and decanting (6 hours). Parameters including total volume, biomass and liquid scintillant cocktail loading will need to be determined prior to construction.

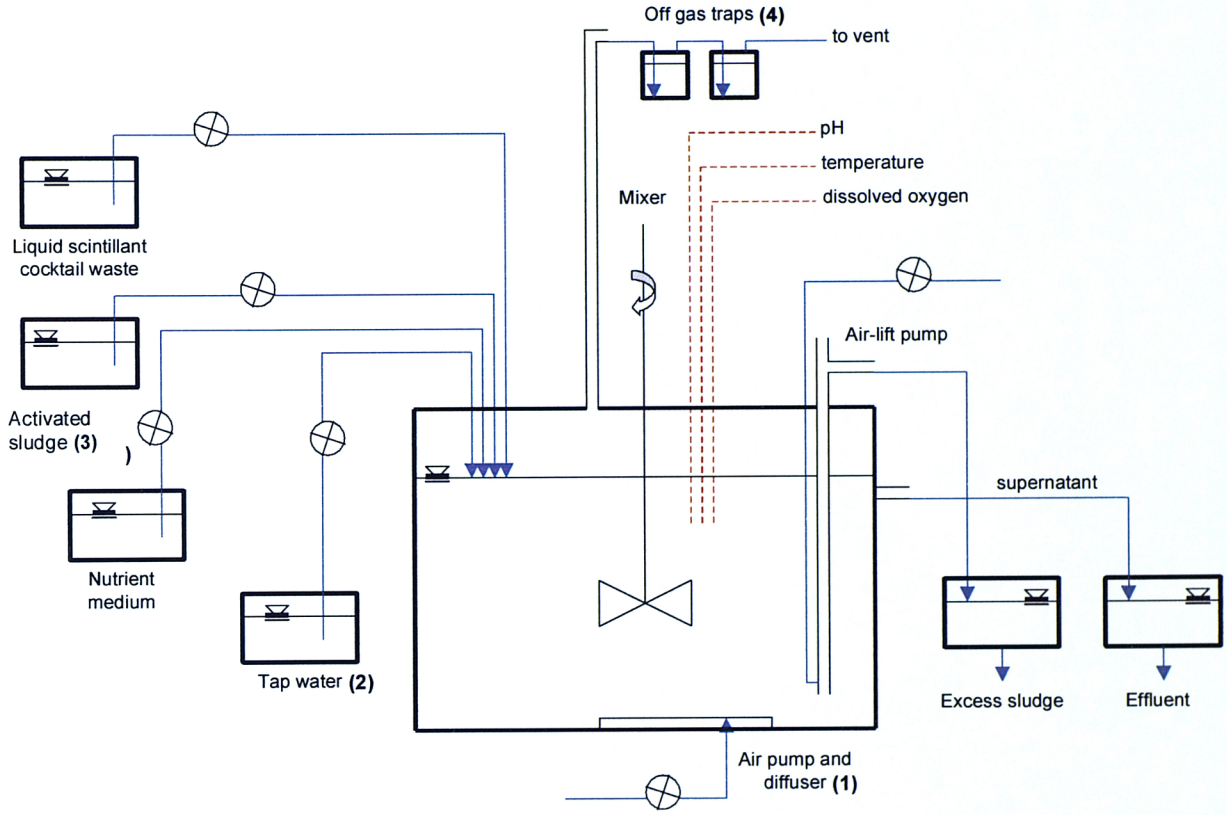


Figure 6.1 Schematic diagram of a pilot scale sequencing batch reactor for the biodegradation of liquid scintillant cocktails

Key: level sensor
pump
flow of liquids
---- continuously monitored parameters

Notes:
(1) air pump and diffuser – aeration to aid mixing and control dissolved oxygen level
(2) tap water – for initial dilution and to maintain level in bioreactor
(3) activated sludge – new sludge every 8 cycles (pre-adapted)
(4) off gas traps – to trap highly volatile components and degradation products

6.2.3.1 Monitoring of the process

On-line monitors are essential in treatment units such as this one, as they provide information on key process parameters, like pH, temperature, liquid level and dissolved oxygen content, and allow for process control (Guwy *et al.*, 1998). As discussed in Chapter 3, pH is a simple means of detecting changes in bioreactor composition. Although this research has detected pH changes, no correlation has yet been established with concentration fluctuations. If a relationship was found between the stage of the biodegradation reaction and the pH profile, this parameter would have provided a cheap and easy mechanism by which to monitor the process, and negate the necessity for costly effluent quality assurance. It must be noted however that pH can only be used as a control parameter for reactions that directly affect it (Al-Ghusain *et al.*, 1994).

6.2.3.2 Foaming

Foaming is an important consideration in aerated bioreactors. Foaming can result in problems with overflowing of the bioreactor contents and may make the stripping of volatile organic degradation products from the bioreactor more difficult (Choori *et al.*, 1998). Biodegradation experiments in this study also encountered this problem, specifically due to the presence of surfactants in the liquid scintillant cocktails. The foaming therefore resulted in aeration rates being controlled. A balance needs to be established between aeration rate and foam height, or a non-foaming agent such as quaternary ammonium-based anti-filament polymers (Hwang & Tanaka, 1998) or bentonite (Stratton *et al.*, 1998) could be added to the bioreactor, following an assessment of its effect on biodegradative losses.

6.2.3.3 Final waste disposal

Disposal of the spent biomass and effluent is the final concern of this scale up process. The system outlined in Figure 6.1 involves a settling stage to separate out the biomass flocs from the effluent prior to draining of the effluent.

Following several biodegradation cycles the spent biomass could be sterilised by a variety of means including microwave irradiation (Ashley & Roach, 1990),

and UV-irradiation (Kolch, 1999). UV-irradiation is extensively used in Europe and the United States of America for the disinfection of drinking water. Short wavelength rays between 200 and 280 nm are generally used to target bacterial and viral DNA of water contaminants. When used, the European Committee for Standardisation recommends a dose of 400 J/m^2 (Kolch, 1999). This can be effectively achieved with the use of an industrial UV-lamp. It is this technique that is likely to be most effective for the scaling up process outlined above. However, tests to determine the optimum ultra-violet biomass sterilisation parameters (duration and impact) would need to be conducted.

Discharge volumes will need to be controlled (Bovard & Candillon, 1960) so as not to have a large impact on the receiving environment and water table below. Even so, when disposal occurs into a watercourse it is essential that good dilution conditions are present and therefore a thorough study of conditions in the river are required prior to any disposal occurring. It should also be noted however, that as a consensus on what constitutes a 'safe release' has not yet been reached, not all requests for microbial biomass releases have been approved (Lindow *et al.*, 1989). Thus it would be very important to be able to ensure a sufficiently high level of disinfection.

6.3 Environmental impact assessment

6.3.1 Introduction, purpose and procedure

An environmental impact assessment (EIA) is a systematic procedure that predicts the environmental consequences of a development process prior to it being established (Anhava & Kolehmainen, 1994; Glasson *et al.*, 1994). It is also a legal requirement for most developments. Although most historical developments have been carried out after some assessment of the impacts, the EIA procedure provides a structured and multidisciplinary framework in which the assessments have to be made. It should be noted however, that although EIAs are generally conducted in several stages (Figure 6.2), there should be a feedback mechanism between these steps. For example, public participation can often be useful in several of the stages. In addition, the necessary steps will vary in order and depending on local laws, with UK legislation omitting the

necessity for consideration of alternatives and post–decision monitoring (Department of the Environment, 1989, as cited in Glasson *et al.*, 1994).

The purpose of an EIA is to aid decision-making regarding granting permission for the development to proceed (Anhava & Kolehmainen, 1994; Folke, 1985), to aid the formulation of development actions, and as an instrument for sustainable development (Glasson *et al.*, 1994). Although they cannot be used as a means for decision-making, they help to outline the tradeoffs involved, and assess increased environmental risks as a result of the development project. Despite many development companies considering the EIA as a time-consuming and costly process, it can often be used by them to highlight certain location or operational changes which may act to minimise their environmental impacts and possibly even their waste disposal quantities and qualities, reflected in lower operational costs.

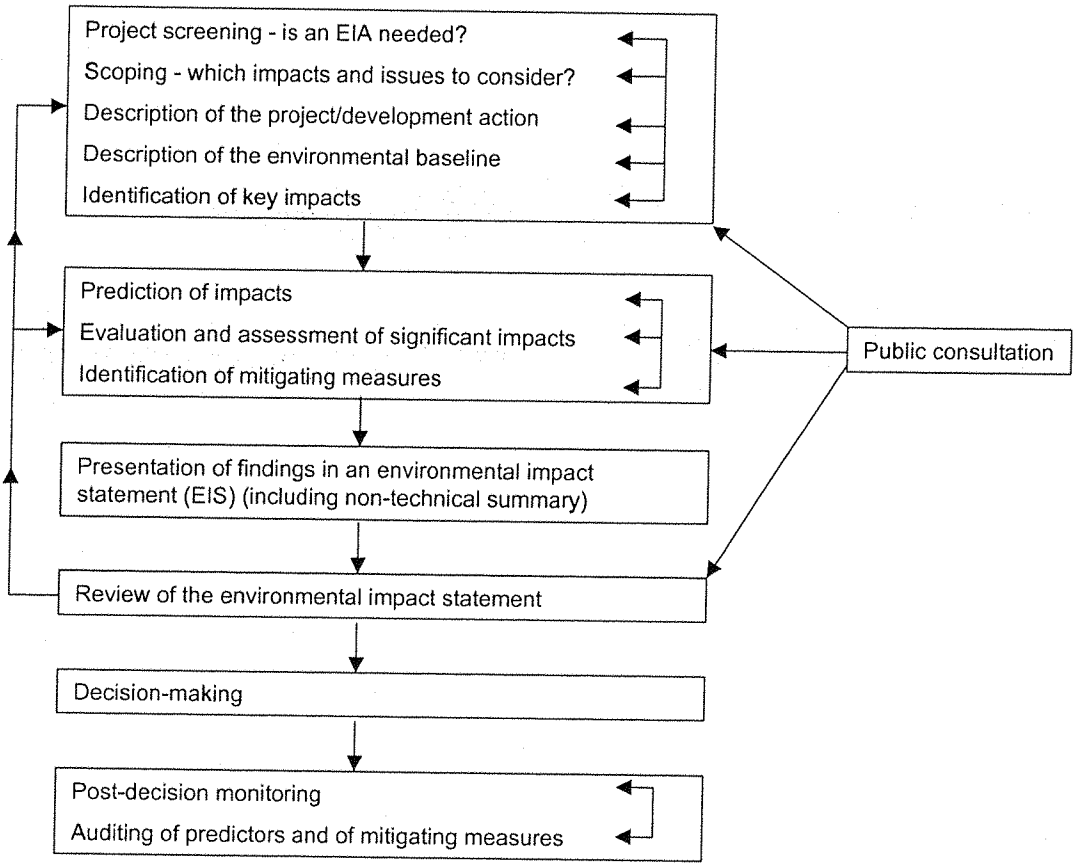


Figure 6.2 Important stages in the EIA process (Source: Glasson *et al.*, 1994)

Legislation regarding EIAs is not only very extensive but varies with location and development type, thus will not be outlined in this discussion. Further details can be found in Glasson *et al.* (1994). The scope of projects covered is ever widening, but is usually considered to be 'major' projects. There is no actual definition for what this category includes, but the scale-up of this project would definitely be considered as such and thus would require an EIA.

6.3.2 Areas of concern for this project

These are likely to be:

- transportation and storage of liquid scintillant cocktail waste,
- use of a biohazard in the form of activated sludge,
- accidental release of liquid scintillant cocktail waste into both the terrestrial and aquatic environment,
- disposal of UV-irradiated spent biomass,
- disposal of effluent, including both non-biodegraded components, purified solutions and a radioactive component, and
- release of gaseous products.

Potential impacts of the accidental release of components of non-processed liquid scintillant cocktails are discussed in Section 2.1. Effluent from the biodegradation process should have far smaller impacts. The biodegradation of some liquid scintillant cocktail components like bis-MSB and emulsifiers (nonylphenol ethoxylates) has not been assessed in this study, and therefore these may be non-biodegradable, hence released in the effluent stream. As nonylphenol ethoxylates are also utilised in large quantities in industrial and domestic surfactants, the relative input from the biodegradation process will be minimal. Even if the surfactants are not biodegraded by the process they should be effectively removed by activated sludge treatment (Matthijs *et al.*, 1997), suggesting that any effluent release is into the wastewater system rather than directly into a watercourse.

The radioactive component of the effluent and biomass to be disposed of will be no greater than that released into the atmosphere by incineration, and will only have an impact on biological systems if it is bioaccumulated in organisms

(Hutchins *et al.*, 1996a). Despite this, all discharges have to be evaluated against a background level (Cook *et al.*, 1998; Hamilton, 1985). Established models, such as that developed by Thiessen *et al.* (1999), are often used for this purpose, with disposal permission only being granted if the process is able to demonstrate that the resultant critical group dose rates fall below the recommended maximum value of 0.3 mSv/year (McDonnell, 1996).

6.3.3 Disposal standards

Important water quality parameters related to the release of effluents include dissolved oxygen, suspended solids, bacteria, nutrients, pH and toxic chemicals (Nyholm, 1992; Tchobanoglous & Burton, 1991), and radioactivity. Regulations regarding wastewater discharge are diverse and subject to change. However, control is generally by effluent standards and discharge permits through the Environment Agency and Water Authorities. Permits are issued on a case-by-case analysis of environmental impacts and specify both effluent quality and quantity, with an aim of meeting receiving water quality standards. It is important that the characteristics of the receiving environment are also taken into consideration in any assessment (Dunster, 1960; Hamilton, 1998), as these will determine how the effluent is further diluted and disposed of.

If toxic chemicals are included in the effluent, then chemical-specific criteria are often outlined in the permit. However, this does not take any synergistic, antagonistic or additive effects of multiple chemicals into account (Tchobanoglous & Burton, 1991). It is therefore sometimes beneficial to use the 'whole-effluent' approach that includes these effects and therefore gives a more realistic assessment of any potential environmental impacts.

6.3.3.1 Effluent tests conducted for this study

A standard technique used to assess whole-effluent quality is biological or chemical oxygen demand (BOD and COD respectively), with an upper threshold limit used to earmark those effluents that should not be directly disposed of into a watercourse. These processes are outlined in Section 3.2.3.2. A BOD and COD assessment was conducted on stage 3 bioreactor samples following a biodegradation period of 52 days. Samples were diluted five-fold in either

distilled water or seawater to determine the effect of these different media on the biomass. If seawater could be used to alter cell structure by osmotic effects, rendering the biomass non-viable, then UV-irradiation would not be necessary, thus reducing operational costs and allowing for direct discharge into an estuarine or marine environment.

COD is a measure of the chemical content of a sample, and thus will not be affected by the viability of the microbial biomass. As expected, the use of seawater for dilution purposes did not have a significant impact on COD levels (Table 6.3). In addition samples from those bioreactors not containing activated sludge (values given in blue), thus not undergoing biodegradation and/or sorption, showed high COD levels. There is no marked difference between EcoSafe™ (ES) and Gold Star™ (GS) bioreactors, suggesting that the extent of biodegradation does not vary markedly as a function of liquid scintillant cocktail used.

Table 6.3 Chemical oxygen demand (COD) values for mixed samples from stage 3 bioreactors following a biodegradation period of 52 days

Bioreactor contents	*COD value for seawater diluted sample (mg/L)*	*COD value for distilled water diluted sample (mg/L)*
ES + distilled water + bactericide + nutrients	4700	4900
ES + distilled water + nutrients	5400	5700
ES + sterilised AS + nutrients	2700	2800
ES + viable AS + nutrients	3900	3800
GS + distilled water + bactericide + nutrients	4600	4700
GS + distilled water + nutrients	4100	4000
GS + sterilised AS + nutrients	2500	2200
GS + viable AS + nutrients	2800	2400

(relative standard deviation of measurement = 1.8 %, level of detection = 100 mg/L)
Note: these values are for the diluted samples only, and have not been multiplied to give true effluent values. Permissable COD level = not applicable

Table 6.4 gives details of the biological oxygen demand (BOD) data. The two data sets correlate well, although the BOD data shows a smaller difference between those bioreactors with and without activated sludge. It would be expected that the use of seawater to dilute the samples would inactivate the

biomass thus reducing biological oxygen demand. This does not appear to have happened in this case, suggesting that the salinity is having little impact on the biomass. Further reductions in BOD and COD levels will occur if the effluent is passed through a wastewater treatment plant, with expected removal levels of 75 - 95 % and 80 - 85 % respectively (Burgess *et al.*, 1999). In addition this will ensure further dilution of the sample, thereby reducing the BOD levels to below the permissible level of around 40 mg/L.

Table 6.4 Biological oxygen demand (BOD_5) values for mixed samples from stage 3 bioreactors following a biodegradation period of 52 days

Bioreactor contents	*BOD_5 value for seawater diluted sample (mg/L)*	*BOD_5 value for distilled water diluted sample (mg/L)*
ES + distilled water + bactericide + nutrients	280	370
ES + distilled water + nutrients	450	250
ES + sterilised AS + nutrients	100	200
ES + viable AS + nutrients	400	380
GS + distilled water + bactericide + nutrients	450	550
GS + distilled water + nutrients	480	430
GS + sterilised AS + nutrients	430	400
GS + viable AS + nutrients	380	310

(relative standard deviation of measurement = 11.7 %, level of detection = 100 mg/L)
Note: these values are for the diluted samples only, and have not been multiplied to give true effluent values. Permissable BOD level = around 40 mg/L (J. Gallop, Environment Agency, *pers comm.*, 2001)

6.3.3.2 Other water quality tests

In addition to BOD and COD tests, other methods of analysing wastewater quality and determining the effectiveness of microbial treatment processes include toxicity testing, such as the Microtox® and *Daphnia* bioassays (Athey *et al.*, 1989, as cited in Aitken *et al.*, 1994; Folke, 1985; Kosmala *et al.*, 1999; Maki *et al.*, 1998). These were found to be very sensitive tests when used for toxicity testing of water samples by Athey and co-workers (1989, as cited in Aitken *et al.*, 1994). Toxicity is also often tested on animals, including earthworms (Dorn & Salanitro, 2000; Potter *et al.*, 1999b), copepods (Thomas *et al.*, 1999), rats and fish (Klein & Gershey, 1990). This list is by no means exhaustive, with the organism used depending on the likely impact group. As different assays can

yield different results (Shuttleworth & Cerniglia, 1995), it is important to conduct several types of toxicity tests. Biodegradability tests and bioaccumulation tests are also sometimes used (Nyholm, 1992). It should be noted that it can be difficult to determine the cause of toxicity if a whole-effluent test is conducted (Nyholm, 1992; Thomas *et al.*, 1999).

6.3.3.3 Obtaining a licence for disposal

It is on the basis of all the environmental impact data that a regulatory body, such as the Environment Agency, will decide whether or not the effluent is of a sufficient quality for discharge into the aquatic environment. With the BOD and COD levels given in the tables above, permission for disposal would not be granted. However, a dilution factor of 50 would reduce these parameters to permissable levels. Following a scale up process, BOD, COD and other parameters such as pH and suspended solids will need to be reassessed as part of an environmental impact assessment prior to any request for disposal permits being made.

The discussion above solely concerns aquatic releases. However, gaseous releases may also be of concern. The scale-up process outlined in Section 6.2 includes a gas trap and therefore aerial discharges will not be significant for this project. If it had been determined through small-scale bioreactor headspace sampling that only very small quantities, if any, of the liquid scintillant cocktail components or degradation products were being lost by volatilisation then a closed system would not be necessary, reducing set-up and maintenance costs of the bioreactors. In this case releases of gaseous products would not be of concern for an environmental impact assessment anyway.

6.4 Commercial viability study

6.4.1 Introduction

Not only would a new process need to be more environmentally friendly than the waste disposal alternatives, but it would also have to have a potential client base and profit margin in order to obtain financial backing for further development.

6.4.2 Commercial viability model

A preliminary model was developed for the scaled-up process outlined in Section 6.2 (Appendix 6). It is a model only and therefore can be used solely to provide an estimate of setting up and running costs, along with an indication of charges that can therefore be made for the disposal service and the profit margins that can be expected. The model assumes the following set operating conditions:

- no temperature control,
- use of a standard nutrient medium,
- single nutrient medium addition,
- the use of activated sludge, which is initially adapted with a liquid scintillant cocktail loading of 5 % for 21 days, and re-used 8 times,
- UV-irradiation to inactivate the biomass prior to disposal, and
- a bioreactor working volume of 1000 L (1 m^3).

For the purposes of cost estimation, further operational variables were set to be:

- a 25 day operational cycle (21 days biodegradation, and 4 days for filling, settling and draining),
- a 15 year life-span of the set up, and
- 300 operational bioreactor days per year.

Table 6.5 outlines bioreactor numbers and costs involved for varying liquid scintillant cocktail loading and disposal market share values. Optimisation of conditions ensures that increases in degradation rate will reduce batch times allowing for greater volumes to be processed in a given time. This in turn would result in an increased return on initial capital costs, further reducing overall costs per unit volume of liquid scintillant cocktail waste processed.

The cost analysis shows that the maximum concentration of liquid scintillant cocktail that can be processed in the bioreactor is the primary contributor to operation costs. For example it is much more economical to operate a single bioreactor at 10 % than ten vessels at 1 % LS cocktail loading. This is due to the high capital costs of the bioreactor systems. This factor is indeed more important that achieving high processing volumes. For example, Table 6.5

shows that processing 10,000 L per annum at 5 % LS cocktail concentration can be achieved at £3.95 /L. This compares with £2.92 /L for 40,000 L at 5 %, a reduction in costs of 26 %. However, if breakdown can be achieved at 20 % concentration, processing costs for 10,000 L drops to £1.82 /L, a saving of 54 %.

Table 6.5 Commercial viability model outcomes with different liquid scintillant cocktail concentration and waste disposal market shares

Liquid scintillant cocktail loading (% by volume)	*1 %*	*5 %*	*5 %*	*5 %*	*10 %*	*20 %*
Total volume processed annually (L)	10,000	10,000	20,000	40,000	10,000	10,000
Number of bioreactors required	84	17	34	67	9	5
Annual set up and maintenance costs of hardware (£)	36,100	9,500	16,300	29,900	6,400	4,800
Annual running costs (£)	105,600	28,900	48,400	8,600	19,900	15,300
Additional annual costs (£)	1,800	1,800	1,800	1,800	1,800	1,800
Liquid scintillant cocktail processing costs (£/L)	**14.23**	**3.95**	**3.26**	**2.92**	**2.59**	**1.82**

Note: Costs do not include labour and transportation costs, or price increases, and are based on estimates

Quotes obtained for LS cocktail incineration range from £ 3.50 – 25 per litre, indicating that bioremediation can be considered an attractive economic as well as environmental option. Atlas (1995), Berktay & Ellis (1997), Hakstege & van Geldermalsen (1998) and Singleton (1994) also considered biological waste treatment as being both feasible and viable.

Chapter 7 - Conclusions and implications

7.1 Conclusions

The work set out to determine break down of the principle components of di-isopropyl naphthalene- (DIPN) and branched alkyl benzene (BAB) based liquid scintillant cocktails through biodegradation.

Experiments involving adaptation of activated sludge biomass to different DIPN based liquid scintillant cocktail (LS cocktail) concentrations were investigated. Due to initial analytical problems, samples taken from the bioreactors could not be analysed for compound concentration, preventing a feedback system being established. Only limited data, in the form of biological oxygen demand and pH values, could therefore be used in these early stages of the study.

Data analysis from these experiments showed:

1. reproducible fluctuations in pH values, the onset of which were a function of adaptation of the biomass,
2. a viable biomass even at 40 % LS cocktail loading,
3. prolonged adaptation of biomass at low LS cocktail concentrations (e.g. 5 %) resulted in higher biomass activity at elevated concentrations than when the biomass had been gradually adapted to increasing concentrations over the full concentration range.

The requirement for further information regarding the biodegradative losses of both the LS cocktails themselves and their individual components resulted in the setting up of an array of twenty-six bioreactors. Several duplicate bioreactor set-ups were included, and aeration rate, pH and temperature were determined in key bioreactors. Samples from these bioreactors were not only used to quantify compounds of interest by GC-MS analysis, but also to investigate biomass growth dynamics and species adaptation by agar plating and optical density measurements.

Microbiological investigations showed that:

1. activated sludge (AS) is both a suitable and inexpensive source of microorganisms for use in biodegradation studies. Fourteen microbial species were identified on the basis of morphological characteristics when

an AS sample was cultivated. Fewer species were present when specific LS cocktail components were used as a sole carbon source suggesting that natural selection was occurring in these cases,

2. at an equivalent compound concentration to that present in a 1 % LS cocktail solution, none of the individual components inhibited biomass growth. This result was also found for both the DIPN-based and BAB-based LS cocktails (Gold Star™ and EcoSafe™ respectively),
3. DIPN, and bis-methylstyryl benzene (bis-MSB) were shown to be unavailable for use as a carbon source, but conflicting outcomes were apparent for the remainder of the components and the LS cocktails themselves, and
4. due to low reproducibility, neither the plating nor optical density measurement techniques could be used for quantitative analysis.

Further details regarding biotic and abiotic losses of LS cocktail components were achieved using an array of eight bioreactors. Varying contents allowed for the determination of losses including those from other sources, possibly including photo-oxidation + volatilisation, sorption, losses due to contamination by airborne microbes and biotic losses. Specific measurement of these mechanisms was not involved, but the bioreactors were set up such that cumulative losses were determined from which contributions of individual loss mechanisms could be deduced. A correlation between microbial growth, as determined by microbiological methods, and biotic concentration losses was apparent suggesting that biodegradation was at least partially attributable to biomass increases.

Reproducibility in the GC-MS data was enhanced by the use of an internal standard (d_8-naphthalene) to compensate for both injection and sensitivity variations. BAB data were further corrected with ratioing to an internally conserved standard (C_{18} BAB with five carbon atoms in its primary aliphatic chain) to correct for errors including heterogeneity in the bioreactor. This correction resulted in useable data. Unfortunately no such second correction was available for the other compounds, and therefore these degradation profiles

were more difficult to interpret. Sampling, liquid-liquid extraction and GC-MS analysis gave a cumulative error of 9 %.

Combined GC-MS results from the 26 bioreactor and 8 bioreactor arrays showed that:

1. the process was reproducible despite a variation in activated sludge composition due to the use of fresh AS, and uncontrolled laboratory conditions used for the experiments. Although loss rates varied slightly, the extent of loss was comparable between experiments,
2. butoxyethoxy ethanol (BEE) and branched alkyl benzenes (BAB) with only one or two carbon atoms in their primary aliphatic chain could be considered biodegradable. Smaller losses, primarily due to abiotic mechanisms, were evident for BAB with longer primary chain lengths. PPO showed total losses within 30 days, which were attributed to biodegradation and substantial abiotic losses. DIPN showed neither biotic nor abiotic losses of any kind,
3. abiotic losses were compound specific, with largest number of loss types for the most readily biodegradable compounds like BEE and the shorter primary aliphatic chain lengthed BAB,
4. compound type was also an important factor in determining lag times, with increased lag durations corresponding to increased recalcitrance. In addition, lag times were dependent on the presence of other compounds and number of loss mechanisms occurring. This was shown by an extended lag phase for PPO in GS in comparison to PPO in ES, suggesting that the DIPN in GS was having an inhibitory effect on PPO losses,
5. first order loss rates too were found to be a function of compound structure, with increased rates correlating with increased susceptibility to biodegradation. The presence of other compounds may reduce loss rates but does not act to prohibit biodegradation, as in the case of PPO in the DIPN-based LS cocktail, and
6. no degradation intermediates or products were detected. It is very unlikely that none was produced, and therefore this was probably an artefact of the sampling or analytical process.

Due to sensitivity and time limitations, bis-MSB could not be quantified and therefore it is uncertain what impact the activated sludge had on this compound. However, the microbiological data showed it to be non-toxic yet unavailable as a growth nutrient. This would therefore suggest that although abiotic losses may occur, biodegradation of this compound is unlikely. Detection of nonylphenol ethoxylates by GC-MS was not possible either. Although their identification and quantification can be achieved by other techniques including electrospray ionisation-mass spectrometry, time and financial restrictions did not allow for this analysis in the scope of this project.

7.2 Implications of the study

The outcomes of this study have certain important implications for the waste disposal of liquid scintillant cocktails. They have shown that butoxyethoxy ethanol (BEE), diphenyl oxazole (PPO) and the shorter primary chained branched alkyl benzenes (BAB) can indeed be reduced in concentration by a combination of biodegradation and abiotic losses. However, the remaining branched alkyl benzene isomers and di-isopropyl naphthalene (DIPN) are not removed by these means. In addition it is unclear what abiotic or biotic losses, if any, occur for bis-methylstyryl benzene (bis-MSB) and nonylphenol ethoxylates (NPEs).

The finding that di-isopropyl naphthalene is not biodegradable at a concentration equal to that in a 1 % liquid scintillant cocktail solution means that DIPN-based cocktails such as Gold Star™, should not be labelled as biodegradable. However, DIPN was shown to be non-toxic to activated sludge and therefore any labelling could only use this term.

It is evident that the biodegradation process suggested in the scale-up cannot be used for DIPN-based liquid scintillant cocktails. Liquid scintillant cocktails containing BAB isomers with primary aliphatic chain lengths of over three carbon atoms cannot be biodegraded by this process either, and biodegradation of bis-MSB and the NPEs has not been determined.

Does this therefore suggest that it is time to develop a new biodegradable liquid scintillant cocktail? This cocktail would need to contain components that have either been shown to be biodegradable, or alternatively at least whose undegraded disposal into the wastewater system will be permitted.

This investigation has shown that DIPN could not be used as such a solvent. However, either a selection of the shorter chained BAB isomers, or a wholly new solvent which can be shown to be biodegradable would be a possible alternative. The use of selected BAB isomers instead of DIPN is unlikely to be popular, as DIPN has been shown to be more efficient at energy transfer than BAB. Therefore, unless the incentives are considered to be sufficient, this switch may be considered to be a regressive step for liquid scintillant measurement (Klein & Gershey, 1990). On the other hand, developing a completely new solvent will both time-consuming and costly.

BEE could be used as an effective co-solvent and PPO fulfils its role as a biodegradable primary fluor. The fate of bis-MSB is unknown. However, this component is not necessary for use with new liquid scintillation counters as the photomultiplier tubes work effectively at the fluorescent wavelengths of primary scintillators like PPO (Kobayashi & Maudsley, 1974). NPEs are a necessary component of LS cocktails, but they are also used in industrial and domestic detergents. As such, large volumes of NPEs are discharged into the wastewater system and treated in sewage treatment plants, and thus there should be no problem obtaining approval for discharge.

This study therefore recommends the development of a new liquid scintillant cocktail containing only the short primary chained BAB, BEE, PPO and NPEs. In this case biodegradation could be used as a viable alternative waste disposal mechanism for the liquid scintillant cocktail waste.

Without further analyses, toxicity testing and a thorough environmental impact assessment, it is uncertain whether or not the products resulting from the biodegradation process will have less environmental impact than those produced by incineration. It is however clear that no particulates are produced,

that carbon dioxide volumes emitted are reduced and that carbon in the liquid scintillant cocktails is partially converted into biomass, which can be disposed of, following sterilisation, into a watercourse. This study also suggests that biodegradation effluent quality will be consistent between batches. This is very important for commercial purposes as both the control of effluent quality and aerial releases would markedly increase operational costs.

The commercial viability study clearly indicates that in order to reduce processing costs per unit volume of liquid scintillant cocktail waste it is more important to increase the liquid scintillant cocktail capacity than market share processed. Any commercial process would require UV-irradiation for final waste disposal of the biomass and effluent.

In addition to the use of this biodegradation technique for cost-effective and environmentally safe disposal of liquid scintillant cocktails it should also be applicable to other organic liquids. With certain modifications and several test experiments, other wastes, including oil contaminated material and certain effluents from chemical processing plants, could be treated by the process developed in this study. Due to its simplicity and the use of easily obtainable materials such as activated sludge and a basic nutrient mix, the technology could also be readily utilised in countries where resources are limited.

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Appendices

A1 Liquid scintillation basics

A1.1 Principles

Liquid scintillant counting can be used to measure alpha, beta and some electron capture radioisotopes.

Energy from radioactive decay in the sample – liquid scintillant mixture, the vial and the apparatus excites the primary solvent molecules. Interaction between these solvent molecules and the primary scintillators results in a transfer of the excess energy to the scintillator molecules. These molecules are raised to an excited energy state, emitting fluorescence as the electrons return to the ground state (Figure A1.1). The emitted photons of light have a wavelength of 350 – 400 nm. In modern scintillation counting systems it is this wavelength that is detected. However, older systems contain less sensitive photomultiplier tubes. A secondary scintillator is therefore used, where the photons emitted by the primary scintillator cause excitation of the secondary scintillator. The energy is re-emitted as a photon of wavelength 400 – 430 nm. In this wavelength range the photomultiplier detection is more efficient, therefore acting to amplify the signal.

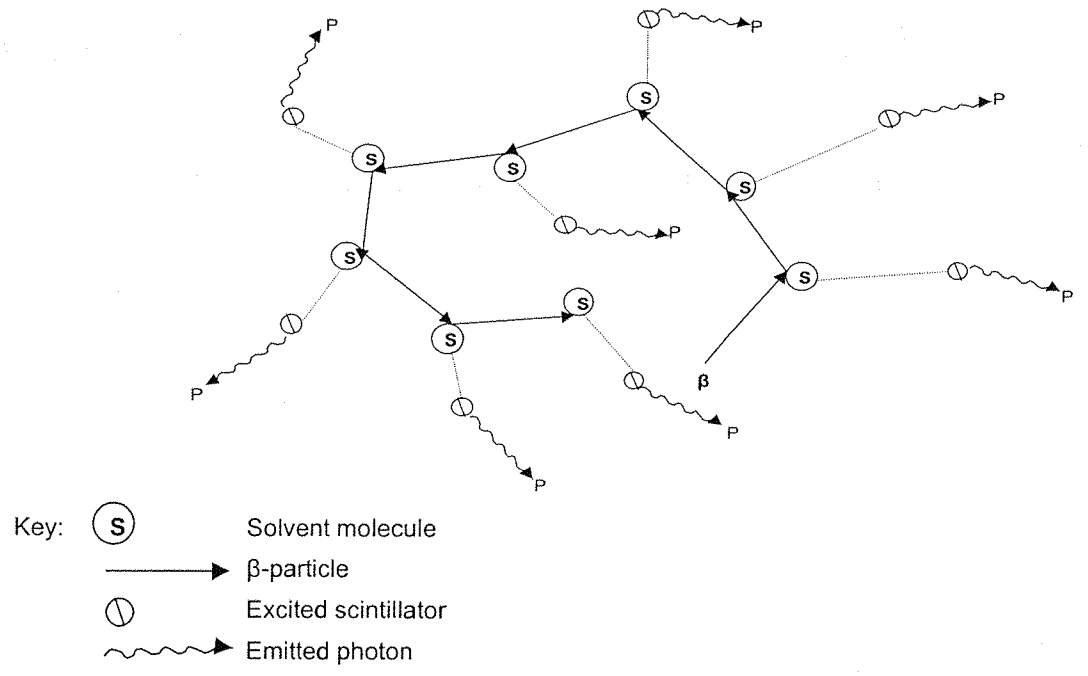


Figure A1.1 The transfer of energy in liquid scintillation counting (adapted from Packard Instrument Company, 1987). Note that a single beta particle is capable of exciting numerous solvent molecules

Light emitted by the scintillator constitutes the scintillation. The number of photons emitted is a function of radioactive content and particle energy, influencing the number of solvent molecules excited. Although solvent molecules do emit photons they are unsuitable for direct scintillant counting as the energy emitted cannot be detected by most photomultiplier systems. In addition, due to the high concentrations of solvent molecules, there is a high probability of this light being re-adsorbed. Increased photomultiplier detection capabilities, and reduced concentrations and fluorescence lifetimes enables enhanced detection of the photons emitted by scintillator molecules.

A1.2 Liquid scintillation counters

Detection of the scintillation occurs when the sample-liquid scintillant cocktail mixture, in a plastic or glass vial, is placed in a light tight enclosure to exclude all cosmic light interferences. A photomultiplier tube is used to quantify the pulses of light emitted by the sample. The alloy-covered photocathode and dynodes in the photomultiplier tubes absorb photons emitted by the scintillators, and convert them to photoelectrons. These pass through several stages of attraction to an electrode and amplification to form a cascade of electrons at the final electrode (Figure A1.1).

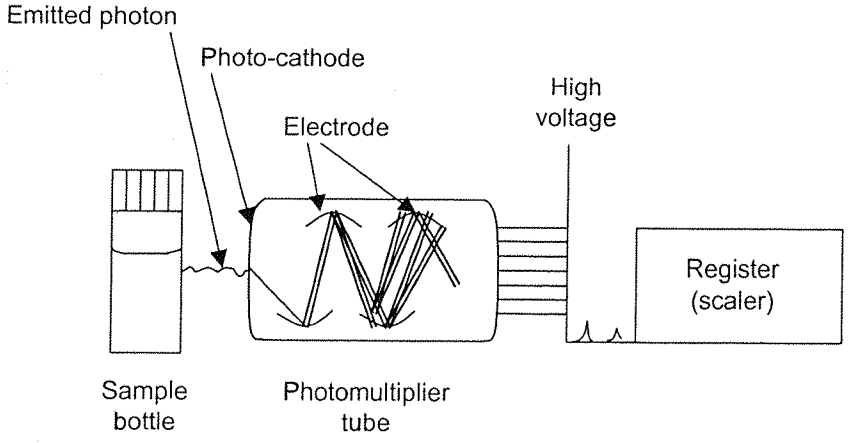


Figure A1.1 Schematic of a liquid scintillation system (adapted from Packard Instrument Company, 1987)

Modern liquid scintillant counters consist of two photomultiplier tubes. The system detects the number of light pulses emitted within a specified time, which

is used to quantify the total activity of the sample. Pulse intensity is also detected to determine the radioactive particle energy that initiated the scintillation process. Some modern systems also incorporate a compensation for background radiation through cosmic events or apparatus contamination by simultaneously measuring the radioactivity of a chamber containing scintillant gel. This is particularly important in the measurement of trace level radionuclides. In addition, separation of the signals from mixtures of radionuclides and some identification can be achieved with the aid of a multi-channel analyser (MCA).

A2 Liquid scintillation cocktail incineration in Slough

Radioactive waste on way to Slough

Report by John Shelley

RADIOACTIVE waste from the Trident nuclear missile programme is earmarked to travel more than 500 miles by road from a Scottish naval base for disposal in Slough.

The Ministry of Defence has applied for permission from environmental watchdogs to incinerate the low level waste in the borough.

The waste, a bi-product of laboratory tests carried out on contaminated drying agent used to pack Trident missile components, is due to be handled by Colnbrook waste company Grundon.

Slough Council has just under two months to consider whether to accept the application from Her Majesty's Naval Base on the Clyde to the borough.

Navy spokesman Steve Willmot said: "There's absolutely no danger to the public but as always we are squeaky clean when it comes to transporting anything which is at all radioactive so anybody who might be concerned has been informed."

Mr Willmot declined to give details of what the tested drying agent had been packed with on the grounds it 'might impinge on national security'.

But he said the chance of developing cancer after coming into contact with the drying agent, scheduled for disposal at a site near Sellafield, Cumbria, was one in two trillion or one in two million million.

No figures were available for how radioactive the liquid waste coming to Colnbrook would be.

"The level we are talking about can't be detected even with the most sensitive equipment that we have," said Mr Willmot.

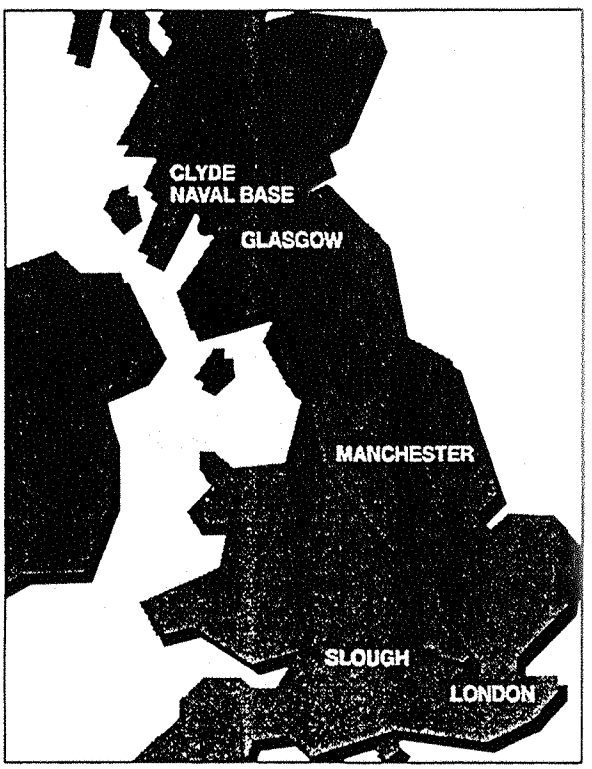
Slough has been chosen to receive the liquid waste, some time after mid-February, because of an existing contract between Lakeside Road company Grundon and scientific supplier Canberra Packard.

Grundon has a licence to take low level radioactive waste and regularly disposes of contaminated products from medical procedures like X-raying.

Grundon's estates director Richard Skehens said: "We often take waste from local hospitals, but we do also take it from further afield from time to time."

The Clyde naval base, on the western coast of Scotland, is home to all four of the UK's nuclear missile submarines and the only remaining nuclear weapons in Great Britain.

Each submarine carries 16 missiles with a range of up to 10,000 miles.



■ *TRIDENT radioactive waste will travel more than 400 miles to be treated at Colnbrook.*

THE EXPRESS Thursday, February 18, 1999 13

Vote to block nuclear waste

COUNCILLORS are being called to vote on moral grounds to block the transfer of nuclear waste from Scotland to their borough.

Slough Borough Council's planning and development committee has been asked to make its views on the proposal to transfer liquid nuclear waste from the Trident military base in Argyll, Scotland to Colnbrook, clear at the next meeting on March 4.

Opposition members, as well as representatives of Colnbrook Parish Council, said it would be wrong of the committee to give its backing to the scheme when the council as a whole is committed to an anti-nuclear stance.

Steven Burkmar (Con: Colnbrook and Poyle), who is a member of the committee which will be discussing the issue, said: "The anti-nuclear stuff is on all the stationery the council sends out. I'm not scaremongering. I quite believe the waste is of low toxicity but it seems as though, yet again, the Labour Group is performing a U-turn."

He confirmed he would be voting against the motion and called on other Labour members of the committee to do the same.

The Scottish Environment Protection Agency has asked for the council's views on the issue although the Ministry of Defence will have the final say on whether the waste will be transferred to the borough.

By GEOFF FOX

If given the go-ahead, the waste will be transported by road to WS Grundon Ltd, Lakeside Road, the country's largest waste disposal company, where it will be incinerated.

Dexter Smith, vice-chairman of Colnbrook Parish Council, said members had decided to campaign against the proposals at a meeting last week and accused Slough Council of hypocrisy if it failed to do the same.

He said: "We don't like being put in the position where we have to pay the social cost."

A spokeswoman for the council admitted there was a conflict of interest because although Grundons already dispose of medical nuclear waste, the proposed scheme would give the firm permission to destroy military waste.

Nigel Dicker, head of Slough council's environmental protection services, said members of the planning and development committee would have to vote on moral and technical grounds, although he explained research carried out by an independent firm for the council alleviated any health fears.

Village protests at Trident waste site

'Help us fight nuclear dump'

PROPOSALS to dispose of radioactive waste from the Trident nuclear missile programme in Colnbrook have been blasted by the parish council.

Colnbrook and Poyle Parish Council says it is crazy to use the village as a waste site for low level radioactive waste brought more than 400 miles from a naval base in Scotland.

And members are worried that plans to incinerate the waste in Slough could be the start of an expansion of Grundon's radioactive waste facility.

Chairman Peter Hood said: "Regardless of what they say, there must be somewhere more appropriate to take it.

"It's such a built-up area with high population density and right next door to a supermarket cold storage facility."

The parish council is writing to the Scottish Environment Protection Agency, objecting to the plans.

Cllr Hood said: "We are asking Slough Borough Council to support us because Slough is a nuclear-free town."

The liquid by-product has been earmarked for disposal in Colnbrook because of an existing contract between waste management company Grundon and scientific suppliers Canberra Packard.

By John Shelley

Grundon's incinerator on the Colnbrook by-pass regularly takes low-level radioactive material from medical procedures such as x-raying.

Steve Willmot, spokesman for the Royal Navy in Scotland, said there was absolutely no danger to the public from the waste.

"The level we are talking about can't be detected even with the most sensitive equipment that we have," he said.

A consultation period on the application – by the Ministry of Defence – ends on Friday, March 5.

Slough's development committee considers the application on Thursday, March 4.

Nuclear waste fears

SCOTTISH authorities will ignore objections from Slough and dispose of radioactive waste at Colnbrook, it is feared.

Colnbrook parish councillors believe the Scottish Environmental Protection Agency will ignore protests and go-ahead with a controversial plan to bring nuclear by-products more than 400 miles from the Trident nuclear missile base in Scotland to Slough.

Last week the borough council agreed to oppose the plans because it has long taken an 'anti-nuclear' stance.

Colnbrook Parish Council has also objected but at a meeting on Tuesday councillors expressed dismay the final decision will not be taken in the town or even the country – a decision will be made in Scotland by SEPA.

Dexter Smith, vice-chairman of Colnbrook and Poyle Parish Council, said: "When I spoke to SEPA it sounded like they had already accepted the waste was coming to Slough."

"The overwhelming feeling I got was that this is a matter they've already made their minds up about.

"The objections from Slough are not likely to carry much weight in Scotland," he added.

HEATHROW SAVED FROM NUCLEAR WASTE

PLANS to burn radioactive waste, from the trident nuclear programme in Scotland, less than a mile from Heathrow have been dropped after protest from local residents.

The radioactive material, tritium from the Royal Naval Armament Depot in Coulport, Dunbartonshire, was due to be burnt at Grundon's incinerator at the Lakeside Industrial estate, Colnbrook on London's borders in Slough, Berkshire once the go ahead had been given this September from the Scottish Environment Protection Agency (SEPA) and the new Scottish Executive.

Grundon, the largest privately owned waste management group in the country, is already licenced to burn domestic and hospital waste, contaminated with low-level radiation, but this proposal was met with shock by local residents who contacted MPs with constituencies bordering the site; persuaded local MP Dominic Grieve to write to the Scottish office and generated intensive coverage in the local media.

"Our site was nominated as one of the few incineration plants of a high enough standard," said Grundon estates director Richard Skehens, "but we have now decided that we do not want to accept this waste. It is a decision which respects the views of our neighbours. The local council, which has a nuclear free policy, and the parish council were both anti it."

"This is a great victory for local democracy," said Dexter Smith, vice-chair of Colnbrook parish council, who helped lead the campaign. "People in the wider world need to know that you can produce results by campaigning and complaining." However he is still concerned about where the Ministry of Defence's radioactive waste is going to end up being disposed, as there are no suitable incinerators in Scotland.

LAST WORD: Scotland's Donald Dewar could still send radioactive waste to London

he whole process is so undemocratic. There is no minister in Westminster who is responsible for this decision because the waste comes from Scotland," complains Smith. "During this proposal the main bodies consulted by the Scottish Environment Protection Agency were based in Scotland except Slough Borough Council and the Environment Agency. This means the First Minister at the Scottish Parliament, Donald Dewar, is at some point going to be asked if it is alright to dispose of this nuclear waste in England; of course he is going to say yes."

Despite turning down the waste from the trident programme, Grundon is still being targeted by local residents. It has recently had a proposal to build on nearby greenbelt land turned down, and as a result it has submitted a new proposal for a much larger energy from waste incinerator at the existing Colnbrook site. A public meeting, organised by Slough Borough Council and the Environment Agency, is due to be held on 23 September at Colnbrook village hall.

A3 Microbial methods

A3.1 Agar and replica plating

Preparation of agar plates:

- 20 g nutrient agar was added to 1 L of Milli-Q^{TM} water in a flask and shaken to dissolve the agar powder
- the flask was plugged with cotton wool and capped with foil to prevent any contamination from liquids outside the vessel
- the agar solution was autoclaved for 15 mins. at 121 °C, and cooled to 50 °C before removing from the autoclave. Alternative carbon sources were added to the cool, but still liquid, autoclaved mixture with the assumption that the analyte mixes were sterile
- approximately 10 mL of the autoclaved mixture was poured into labelled plates following sterilisation of the neck of the flask in a Bunsen burner flame
- plates were closed and left to set at room temperature, following which they were either be used or wrapped in clingfilm and stored inverted in the refrigerator at 4 °C for a maximum of three months

Plating of the sample:

- 9 mL of Ringers solution in small pyrex bottles was autoclaved under the standard conditions mentioned above
- after being left to cool to room temperature, 1 mL activated sludge was added to the first bottle and mixed, resulting in a 1 in 10 dilution
- 1 mL diluted solution was then added to the second bottle of autoclaved Ringers solution resulting in a 1 in 100 dilution. This process was repeated to give tenfold serial sample dilutions up to 1 in 100 000
- 0.1 mL of each diluted sample was transferred onto a plate. The liquid was spread over the surface of the gel using a sterilised angular glass rod. The glass rod was sterilised between each sample by dipping in methylated spirits, flaming in a Bunsen burner and leaving it to cool prior to spreading the next sample. Several blanks were also made up to assess environmental contamination, using sterilised Milli-Q^{TM} water and no plated sample

- the plates were closed, inverted, wrapped in clingfilm and stored at room temperature in the dark for 7 days

Replica plating:

- only agar plates where 30 – 50 colonies were present were used to ensure minimal smudging the transfer of sufficient colonies
- a sterile velvet pad was stretched over the frame and pressed lightly onto the agar plate colonies
- the pad was then gently pressed onto the new and other replicate plates in quick succession. A single dab on the original plate was found to be sufficient for the transfer of colonies for up to 10 new agar plates. Blanks were included where a clean velvet pad was pushed onto a new plate to assess pad contamination, and where no contact with a pad occurred at all to assess environmental contamination
- as before, following closing of the plates, they were inverted, wrapped in clingfilm and stored at room temperature in the dark for 7 days

Bacterial enumerations:

- following a culture period of 7 days the plates were unwrapped and colonies were counted by marking on the underside of the plate using a permanent marker
- in several cases counting for the 1 in 10, 1 in 100 and 1 in 100 000 diluted plates was impossible due to either a surplus or absence of colonies
- results were recorded as colony forming units per mL

All work was carried out with a minimum of three replicates. Sterile pipette tips and a sterile laminar flow cabinet were used in all cases. Ringers solution was used for dilution purposes as an isotonic medium to ensure the maintenance of cell integrity during the procedure. When the plates had been counted and were no longer required for further replica plating, they were placed in open autoclave bags and autoclaved under the standard conditions described above. Once the bags had cooled they were sealed and disposed of as non-hazardous waste.

A3.2 Optical density measurements

Stock preparation:

- *nutrient mix stock* was prepared using 5 mL of each of the nutrient concentrates and UV-irradiated Milli-Q™ water to make up to a total volume of 500 mL (Table A3.1).

Table A3.1 Details of nutrient stock solutions

Stock solution number	*Constituent (s)*	*Mass (g) per 100 mL*
1	$Ca(NO_3)_2$	2.00
2	KH_2PO_4	1.24
3	$MgSO_4.7H_2O$	5.00
4	$NaHCO_3$	1.59
5	EDTAFeNa $EDTANa_2$	Combined = 0.225
6	H_3BO_3 $MnCl_2.4H_2O$ $(NH_4)_6MO_7O_{24}.4H_2O$	0.258 0.139 0.10
7	Vitamin mix of B_1 and B_{12}	unknown
8	$NaNO_3$	8.00
9	$Na_2HPO_4.12H_2O$	3.60

- *individual analyte stocks* were made up at ten times the normal 1 % LS cocktail concentration equivalent (w/v) by using the following amounts in a total volume of 100 mL with UV-irradiated Milli-Q™ water.

Bis-methylstyryl benzene (Bis-MSB)	1.712 g
Butoxyethoxyethanol (BEE)	1.119 g
Di-isopropyl naphthalene (DIPN)	35.000 g
Diphenyl oxazole (PPO)	0.007 g
Gold Star™ (GS)	10.000 mL
EcoSafe™ (ES)	10.000 mL

- *general carbon stock* was also prepared using 15 g/L of sucrose in UV-irradiated Milli-Q™ water.

General sample preparation:

- the following solutions were added to a 15 mL sterilised glass sealable vessel:

  1 mL concentrated nutrient mix stock
  1 mL general carbon stock
  4 mL biomass (either AS or bioreactor sample)
  1 mL of the required individual analyte stock (depending on experiment), and
  UV-irradiated Milli-Q^{TM} water to a total volume of 10 mL

- the vessels were sealed with an autoclaved foam bung and foil cap and place on a shaker at 80 rpm and 24 °C for 3 days to allow time for colony development

Calibration experiments:

- final broth solution was diluted 1 in 2, 1 in 5, 1 in 10, 1 in 20, and 1 in 40
- spectrophotometer was left for to warm up for 30 mins. prior to zeroing at a wavelength of 600 nm
- samples were shaken to thoroughly mix and resuspend the contents and a subsample was poured into a quartz cuvette, placed in the light beam path of the spectrophotometer, and a reading of both absorbance and transmission was taken and noted
- between each sample the cuvette was washed out thoroughly in triplicate using UV-irradiated Milli-Q^{TM} water

Microorganism enumerations:

- the Leitz Diaplan microscope was set up with Image Pro plus software to facilitate cell viewing on a computer monitor with a 100 x magnification
- a sheet of clear plastic was attached to the monitor
- using a clean Pasteur pipette, a subsample was taken from the diluted broth and place on a calibrated grid (graticule) with a clean cover slip for counting
- counts were made for ten randomly selected squares by marking the cells on the plastic sheet using a marker pen. Each square had a volume of 5 x 10^{-8} mL

- the graticule was cleaned between each sample using UV-irradiated Milli-Q™ water and was dried with a lens tissue to prevent dilution of the following sample

Growth experiments to answer the research questions:

- all of these experiments were carried out in triplicate
- nutrient stock and analyte solution combinations used are given in Table A3.2
- optical density measurements were carried out on day 0 and day 3 following cultivation under standard conditions, zeroing the spectrophotometer with UV-irradiated Milli-Q™ water. Distilled water was also used for this purpose by Ahn *et al.* (1998) and (1999)

Several of the experiments were prepared and analysed in triplicate to determine procedural and analytical errors.

A4 GC-MS methods

The aim of this appendix is to provide a detailed description of the sample preparation and analytical details for GC-MS analysis.

A4.1 Liquid-liquid extraction and sample dilution

- the sample was defrosted overnight in a fume cupboard, out of direct sunlight
- it was then thoroughly homogenised using an autovortex for 10 seconds
- into a clean, labelled glass liquid scintillation vial 2 mL sample, 3 mL Milli-Q^{TM} water and 5 mL dichloromethane were pipetted
- 10 drops of 1.2 M hydrochloric acid were added, the vial was sealed and rocked by hand for 1 minute
- the vial was then left to stand, allowing the organic and aqueous phases to separate
- 1 mL of the bottom (organic) phase was pipetted into a clean, labelled scintillation vial and the remainder of the organic phase was removed to waste, leaving only the aqueous phase
- a further 5 mL of dichloromethane was added to the aqueous sample, the vial was sealed, rocked, and left to separate
- another 1 mL subsample of the solvent phase was added to the first and the extraction was repeated, resulting in a total of three extractions, with the solvent aliquots being combined
- to the combined extracts with a total volume of 3 mL, 1 mL of 20 ppm d_8-naphthalene in dichloromethane was added resulting in a final sample dilution of 1 in 10 and d_8-naphthalene concentration of 5 ppm
- immediately prior to analysis, 1 mL dichloromethane was pipetted into a hypervial, 100 µL were removed and a 100 µL sample extract aliquot was added, resulting in a final sample dilution of 1 in 100 and a d_8-naphthalene internal standard concentration of 0.5 ppm.

All of this work was carried out in a fume cupboard with full protective clothing. In addition, new or thoroughly cleaned liquid scintillation vials and pipette tips were used. Any unused sample was returned to the freezer at a temperature of –4 °C for storage in the case of further analysis being required. All labelling

consisted of batch number, bioreactor number and degradation day in the form of A-B-C, where:

A = Batch number

B = Bioreactor number

C = Degradation day

A4.2 GC-MS operation conditions

Table A4.1 GC-MS operating parameters

GC-MS section	*Parameter*	*Operating conditions*
GC injector	- injection mode - initial split flow - initial time - purge mode - purge flow rate - purge duration - temperature	- splitless - 0 mL/min. - 1 min. - split - 60 mL/min. - 5 mins. - 250 °C
GC Oven	- gas flow rate - initial temperature - initial time - ramp rate - final temperature - final time	- 1.2 mL/min. - 50 °C - 3 mins. - 10 °C/min. - 300 °C - 7 mins.
Mass spectrometer	- transfer line temperature - source temperature - ionisation method - detector voltage - acquisition type - scan range - scan rate - solvent delay time	- 250 °C - 200 °C - Electron impact - 350 V - full scan - 50–590 Da - 2 scans/sec. - 3 mins.

A4.3 Analytical procedure

- a 1 µL aliquot of testmix was first analysed for apparatus sensitivity, the value of the naphthalene peak area was recorded for quality assurance purposes
- with sufficient sensitivity as shown by the testmix results, the extracted samples were then randomly analysed
- d_8-naphthalene peak area was determined and either remedial action was taken or the next sample was injected and analysed. Solvent blanks and triplicate analysis were conducted in the same manner

- following analysis, the batch of sample data was processed using the parameters given in Table A4.2. Manual selection of peaks resulted in the integration of a selected peak, the peak area of which was recorded
- data was input into a database and internal standard corrected. Further processing depended on the compound concerned
- the chromatogram was also checked for the appearance of any possible degradation product peaks

Table A4.2 Processing method parameters

Parameter	
Area noise factor	5
Peak detection	to nearest retention time
Minimal signal/noise ratio	3
Retention times reference peak	D_8-naphthalene

Retention time reproducibility

peak area retention times for d8-naphthalene peak

Date	number of samples	relative standard deviation
4/3/00	27	0.05
4/4/00	10	0.03
4/10/00	35	0.06
4/11/00	24	0.05
4/15/00	30	0.05
4/18/00	14	0.06
4/25/00	10	0.05
4/26/00	30	0.07
4/27/00	30	0.09
	average	**0.06**

average RSD (n = 210) = 0.06

analytical reproducibility

retention time	sample/TIC peak area injection 1	injection 2	injection 3	injection 4	average	stdev	**RSD (%)**
9.75	23,266,439	22,524,194	21,622,575	22,636,708	22,512,479	677,238	**3.0**
10.7	18,645,966	17,009,532	16,795,556	16,624,709	17,268,941	931,418	**5.4**
11.05	24,072,402	21,312,908	22,833,216	23,085,537	22,826,016	1,141,691	**5.0**
14.02	25,259,082	24,697,652	24,272,351	24,679,583	24,727,167	405,350	**1.6**
16.54	25,261,916	25,817,623	24,899,799	25,126,545	25,276,471	390,477	**1.5**

these peak areas are for 5 peaks in the SGE Textmix C injected 4 times

1 % Gold Star bioreactor data

key: m/z = mass-to-charge ratio of compound of interest
ISTD = internal standard (d8-naphthalene)
RSD = relative standard deviation

BEE = butoxyethoxyethanol
DIPN 1 = di-isopropyl naphthalene isomer 1
DIPN 2 = di-isopropyl naphthalene isomer 2
PPO = diphenyl oxazole

sample name	extraction details	degradation day	d8-naphthalene m/z=136	BEE m/z=57	BEE ISTD corrected	BEE % of original concentration	DIPN 1 m/z=197	DIPN 1 ISTD corrected	DIPN 1 % of original concentration	DIPN 2 m/z197	DIPN 2 ISTD corrected	DIPN 2 % of original concentration	PPO m/z=221	PPO ISTD corrected	PPO % of original concentration
A-26-0		0	610,222	2,536,192	2,483,030	**84**	2,448,668	2,397,341	**83**	2,863,729	2,803,702	**84**	151,296	148,125	**82**
A-26-0		0	852,406	4,707,776	3,299,568	**112**	4,153,515	2,911,099	**101**	4,811,208	3,372,061	**101**	243,269	170,501	**95**
A-26-0		0	215,479	1,105,597	3,065,347	**104**	1,197,786	3,320,948	**115**	1,372,811	3,806,217	**114**	79,455	220,295	**123**
	average of day 0 values				**2,949,315**	**100**		**2,876,463**	**100**		**3,327,327**	**100**		**179,640**	**100**
A-26-2	A	2	206,757	210,496	608,235	**21**	1,179,659	3,408,663	**119**	1,397,318	4,037,595	**121**	52,646	152,122	**85**
A-26-2	B	2	177,601	156,562	526,658	**18**	1,060,415	3,567,124	**124**	1,275,310	4,290,008	**129**	47,114	158,487	**88**
A-26-2	C	2	160,824	142,601	529,736	**18**	912,297	3,389,012	**118**	1,021,177	3,793,481	**114**	41,092	152,649	**85**
A-26-2	C	2	169,021	204,795	723,880	**25**	917,474	3,242,954	**113**	1,117,239	3,949,055	**119**	34,095	120,514	**67**
A-26-2	C	2	211,037	192,073	543,745	**18**	1,159,785	3,283,270	**114**	1,410,856	3,994,035	**120**	51,183	144,895	**81**
A-26-6		6	223,724	215	574	**0**	1,496,409	3,996,000	**139**	1,760,098	4,700,153	**141**	83,540	223,085	**124**
A-26-9		9	171,738	60	209	**0**	969,502	3,372,641	**117**	1,114,292	3,876,327	**116**	39,873	138,708	**77**
A-26-13ii		13	156,761	90	343	**0**	963,926	3,673,613	**128**	1,124,484	4,285,515	**129**	113	431	**0**
A-26-16	A	16	158,470	2,630	9,915	**0**	992,216	3,740,649	**130**	1,173,209	4,422,991	**133**	51	192	**0**
A-26-16	A	16	136,966	1,027	4,480	**0**	912,772	3,981,414	**138**	1,088,620	4,748,444	**143**	129	563	**0**
A-26-16	A	16	182,168	1,970	6,461	**0**	1,223,266	4,011,775	**139**	1,451,851	4,761,433	**143**	95	312	**0**
A-26-16	B	16	176,396	426	1,443	**0**	1,114,501	3,774,674	**131**	1,303,009	4,413,127	**133**	87	295	**0**
A-26-16	C	16	188,416	1,129	3,580	**0**	1,201,374	3,809,327	**132**	1,432,275	4,541,469	**136**	43	136	**0**
A-26-23i		23	193,483	75	232	**0**	787,781	2,432,487	**85**	1,040,323	3,212,278	**97**	82	253	**0**
A-26-23iii		23	200,134	136	406	**0**	1,083,510	3,234,445	**112**	1,281,238	3,824,694	**115**	106	316	**0**
A-26-30 ii		30	125,596	428	2,036	**0**	754,706	3,589,961	**125**	893,531	4,250,319	**128**	33	157	**0**

average ISTD peak area used for all calculations **597,431**

day 0 samples	extracted on different days		average	2,949,315	2,876,463	3,327,327	179,640
			stdev	420,453	462,777	502,753	36,943
			RSD (%)	14.3	16.1	15.1	20.6
			incorporates storage, sampling, extraction and analytical variations				
day 2	extracted in triplicate and analysed in triplicate	extraction repro	average	586,451	3,378,205	4,012,835	145,733
			stdev	83,631	126,531	180,261	14,900
			RSD (%)	14.3	3.7	4.5	10.2
			incorporates sampling, extraction and analytical variations				
		analytical repro.	average	599,120	3,305,079	3,912,190	139,353
			stdev	108,272	75,432	105,236	16,769
			RSD (%)	18.1	2.3	2.7	12.0
			incorporates analytical variations only				
day 16	extracted in triplicate and analysed in triplicate	extraction repro	average	5,176	3,863,568	4,577,493	300
			stdev	3,205	124,304	169,737	164
			RSD (%)	61.9	3.2	3.7	54.7
			incorporates sampling, extraction and analytical variations				
		analytical repro.	average	6,952	3,911,279	4,644,289	356
			stdev	2751	148548	191760	189
			RSD (%)	39.6	3.8	4.1	53.2
			incorporates analytical variations only				

1 % BAB bioreactor data

key: Rt = retention time of isomer
ISTD = internal standard (d8-naphthalene)
RSD = relative standard deviation
BAB = branched alkyl benzene
data is given for all the detected isomers of a BAB with 19 carbon atoms

		d8-naphthalene	**BAB peak 15**		(1-pentyloctyl benzene)	**BAB peak 16**		(1-butylnonyl benzene)		**BAB peak 17**		(1-propyldecyl benzene)	
			Rt = 19.05	ISTD	assumes no	Rt = 19.12	ISTD	ratioed to ISTD	*% of original*	Rt = 19.26	ISTD	ratioed to ISTD	*% of original*
sample	degr. day	m/z=136	peak area	corrected	change w. time	peak area	corrected	corrected peak 15	*concentration*	peak area	corrected	corrected peak 15	*concentration*
A-9-0	0	347,901	1,868,842	3,209,258	1.000	1,175,462	2,018,555	0.629	*100*	823,214	1,413,660	0.440	*100*
A-9-2	2	215,696	1,602,488	4,438,543	1.000	1,043,892	2,891,354	0.651	*104*	764,326	2,117,017	0.477	*108*
A-9-6	6	202,244	1,487,970	4,395,480	1.000	948,415	2,801,628	0.637	*101*	713,218	2,106,854	0.479	*109*
A-9-9	9	237,077	3,165,765	7,977,687	1.000	1,952,925	4,921,346	0.617	*98*	1,434,100	3,613,914	0.453	*103*
A-9-9	9	248,513	2,666,464	6,410,241	1.000	1,667,306	4,008,242	0.625	*99*	1,243,512	2,989,432	0.466	*106*
A-9-13	13	179,005	1,910,062	6,374,851	1.000	1,189,762	3,970,843	0.623	*99*	851,493	2,841,867	0.446	*101*
A-9-16	16	198,049	1,350,373	4,073,511	1.000	824,196	2,486,255	0.610	*97*	559,621	1,688,142	0.414	*94*
A-9-23	23	197,353	1,639,819	4,964,093	1.000	981,087	2,969,966	0.598	*95*	600,634	1,818,251	0.366	*83*
A-9-30	30	313,042	2,199,527	4,197,729	1.000	1,310,860	2,501,736	0.596	*95*	797,343	1,521,705	0.363	*82*
A-9-49	49	209,751	1,679,072	4,782,479	1.000	982,472	2,798,362	0.585	*93*	582,784	1,659,936	0.347	*79*
A-9-73	73	189,341	1,272,398	4,014,820	1.000	724,829	2,287,066	0.570	*91*	417,583	1,317,607	0.328	*75*

average ISTD
peak area used **597,431**
used for all calculations

		d8-naphthalene	**BAB peak 18**		(1-ethylundecyl benzene)		**BAB peak 19**		(1-methyldodecyl benzene)	
			Rt = 19.52	ISTD	ratioed to ISTD	*% of origina*	Rt = 19.94	ISTD	ratioed to ISTD	*% of original*
sample	degr. day	m/z=136	peak area	corrected	corrected peak 15	*concentrati*	peak area	corrected	corrected peak	*concentration*
A-9-0	0	347,901	535,247	919,150	0.286	*100*	97,583	167,574	0.052	*100*
A-9-2	2	215,696	525,830	1,456,435	0.328	*115*	90,863	251,671	0.057	*109*
A-9-6	6	202,244	484,155	1,430,199	0.325	*114*	77,399	228,637	0.052	*100*
A-9-9	9	237,077	948,915	2,391,254	0.300	*105*	115,093	290,033	0.036	*70*
A-9-9	9	248,513	865,003	2,079,487	0.324	*113*	101,601	244,251	0.038	*73*
A-9-13	13	179,005	427,706	1,427,473	0.224	*78*	4,085	13,634	0.002	*4*
A-9-16	16	198,049	197,858	596,855	0.147	*51*	2,397	7,231	0.002	*3*
A-9-23	23	197,353	180,854	547,485	0.110	*39*	1,044	3,160	0.001	*1*
A-9-30	30	313,042	169,006	322,543	0.077	*27*	2,037	3,888	0.001	*2*
A-9-49	49	209,751	170,326	485,137	0.101	*35*	1,859	5,295	0.001	*2*
A-9-73	73	189,341	97,126	306,463	0.076	*27*	1,301	4,105	0.001	*2*

BATCH A DATA

1 % BEE bioreactor data (rig 1)

sample name	extraction details	degr. day	BEE ISTD corrected	% of original concentration
A-8-0B		0	9,996,281	**84**
A-8-0	extracted 31/10	0	13,689,617	**116**
A-8-2	extracted 31/7	2	20,719,179	**175**
A-8-2	extracted 31/7	2	21,367,339	**180**
A-8-2	extracted 31/10	2	14,319,147	**121**
A-8-6	extracted 31/10	6	112,004	**1**
A-8-9		9	25,506	**0**
A-8-9	extracted 26/10	9	1,000	**0**
A-8-9	extracted 26/10	9	532	**0**
A-8-13	extracted 26/10	13	1,234	**0**
A-8-16	extracted 31/7	16	456	**0**
A-8-20		20	4,757	**0**
A-8-30	extracted 26/10	30	501	**0**

1 % BEE bioreactor control (not including ASS)

		degr. day	BEE ISTD corrected	% of original concentration
A-6-0	BEE control	0	9,647,269	**87**
A-6-0	extracted 31/10	0	15,670,967	**141**
A-6-2	extracted 26/10	2	16,054,998	**144**
A-6-6	extracted 26/10	6	14,090,619	**126**
A-6-9	extracted 31/7/0(	9	18,077,727	**162**
A-6-9	extracted 31/7/0(	9	17,924,304	**161**
A-6-9	extracted 31/7/0(	9	18,475,429	**166**
A-6-9	extracted 31/7/0(	9	17,250,112	**155**
A-6-13	extracted 26/10	13	11,204,474	**101**
A-6-20		20	7,248,683	**65**
A-6-23	extracted 26/10	23	7,579,972	**68**
A-6-30	extracted 26/10	30	3,912,768	**35**
A-6-49	extracted 31/10	49	478	**0**

1 % PPO bioreactor data (rig 1)

		degr. day	PPO ISTD corrected	% of original concentration
A-12-0		0	441,727	**100**
A-12-6	extracted 31/10	6	6,759	**2**
A-12-9		9	304	**0**
A-12-9	extracted 31/10	9	86	**0**
A-12-13	extracted 31/10	13	6,338	**1**
A-12-20	extracted 31/10	20	48,575	**11**
A-12-23	extracted 31/10	23	161,366	**37**
A-12-27	extracted 31/7/0(	27	25,098	**6**
A-12-27	extracted 31/7/0(	27	24,338	**6**
A-12-30		30	939	**0**

1 % DIPN bioreactor (rig 1)

		degr. day	DIPN 1 ISTD corrected	% of original concentration	DIPN 2 ISTD corrected	% of original concentration
A-18-0	extracted 26/10	0	4,056,848	**100**	4,745,033	**100**
A-18-9	extracted 26/10	9	5,275,819	**130**	6,113,708	**129**
A-18-16	extracted 26/10	16	8,120,486	**200**	9,394,253	**198**
A-18-20	extracted 26/10	20	7,828,479	**193**	9,184,201	**194**
A-18-28	extracted 26/10	28	8,782,952	**216**	10,613,779	**224**
A-18-30		30	4,996,390	**123**	5,501,889	**116**

1 % GS bioreactor (rig 1)

			BEE		DIPN 1		DIPN 2		PPO	
		degr. day	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration
A-26-0		0	2,483,032	**84**	2,397,342	**83**	2,803,703	**84**	148,125	**82**
A-26-0		0	3,299,570	**112**	2,911,101	**101**	3,372,063	**101**	170,502	**95**
A-26-0	extracted 31/10	0	3,065,349	**104**	3,320,950	**115**	3,806,219	**114**	220,295	**123**
A-26-2	extraction A 12/9	2	608,235	**21**	3,408,665	**119**	4,037,598	**121**	152,122	**85**
A-26-2	extraction B	2	526,658	**18**	3,567,126	**124**	4,290,011	**129**	158,487	**88**
A-26-2	extraction B	2	40,382	**1**	4,189,080	**146**	4,804,692	**144**	261,528	**146**
A-26-2	extraction C	2	529,736	**18**	3,389,014	**118**	3,793,483	**114**	152,649	**85**
A-26-2	extraction C	2	723,880	**25**	3,242,956	**113**	3,949,057	**119**	120,514	**67**
A-26-2	extraction C	2	543,746	**18**	3,283,272	**114**	3,994,037	**120**	144,896	**81**
A-26-6		6	574	**0**	3,996,002	**139**	4,700,156	**141**	223,085	**124**
A-26-6		6	28,635	**1**	4,760,249	**165**	5,539,701	**166**	294,435	**164**
A-26-9	extracted 31/10	9	209	**0**	3,372,643	**117**	3,876,329	**116**	138,708	**77**
A-26-13ii	extracted 31/10	13	343	**0**	3,673,615	**128**	4,285,517	**129**	431	**0**
A-26-16	extraction A	16	9,915	**0**	3,740,651	**130**	4,422,994	**133**	192	**0**
A-26-16	extraction A	16	4,480	**0**	3,981,416	**138**	4,748,447	**143**	563	**0**
A-26-16	extraction A	16	6,461	**0**	4,011,777	**139**	4,761,436	**143**	312	**0**
A-26-16	extraction B	16	1,443	**0**	3,774,677	**131**	4,413,130	**133**	295	**0**
A-26-16	extraction C	16	3,580	**0**	3,809,329	**132**	4,541,472	**136**	136	**0**
A-26-23i		23	232	**0**	2,432,488	**85**	3,212,280	**97**	253	**0**
A-26-23iii	extracted 31/10	23	406	**0**	3,234,447	**112**	3,824,696	**115**	316	**0**
A-26-30 ii		30	2,036	**0**	3,589,963	**150**	4,250,322	**128**	157	**0**

1 % ES control bioreactor (containing no ASS)

			PPO		
		degr. day	ISTD corrected	% of original concentration	
A-1-0	ES control	0	96,797	**72**	
A-1-0	extracted 31/10	0	171,743	**128**	contains no BEE or DIPN
A-1-6	extracted 31/10	6	129,800	**97**	
A-1-9		9	193,555	**144**	
A-1-20		20	325	**0**	
A-1-20		20	192	**0**	
A-1-30		30	280	**0**	
A-1-30		30	438	**0**	

1 % ES bioreactor (rig 1)

			PPO		
		degr. day	ISTD corrected	% of original concentration	
A-9-0	ES 1	0	61,867	**100**	
A-9-2	extracted 31/10	2	145,638	**235**	
A-9-6	extracted 31/10	6	201	**0**	contains no BEE or DIPN
A-9-9		9	181	**0**	
A-9-9		9	101	**0**	
A-9-13	extracted 31/10	13	260	**0**	
A-9-16	extracted 31/10	16	202	**0**	
A-9-23		23	176	**0**	
A-9-30		30	160	**0**	
A-9-49		49	197	**0**	
A-9-73		73	224	**0**	

BATCH B DATA

1 % ES bioreactor (possible losses include photo-oxidation and volatilisation)

sample name	degr. day	PPO ISTD corrected	% of original concentration
B-19-0 (1)	0	195,757	**100**
B-19-0 (2)	0	180,728	**92**
B-19-0 (3)	0	199,544	**102**
B-19-4	4	130,667	**67**
B-19-9	9	219	**0**
B-19-14	14	238	**0**
B-19-21	21	118	**0**
B-19-29	29	134	**0**

1 % ES bioreactor (possible losses include those due to airborne contamination, photo-oxidation and volatilisation)

sample name	degr. day	PPO ISTD corrected	% of original concentration
B-20-0	0	203,973	**100**
B-20-4	4	114,552	**56**
B-20-9	9	35	**0**
B-20-14	14	190	**0**
B-20-21	21	125	**0**
B-20-29	29	612	**0**

1 % ES bioreactor (possible losses include all abiotic losses)

sample name	degr. day	PPO ISTD corrected	% of original concentration
B-21-0	0	213,594	**100**
B-21-4	4	111,711	**52**
B-21-9	9	52	**0**
B-21-14	14	384	**0**
B-21-21	21	65	**0**
B-21-29	29	3,042	**1**

1 % ES bioreactor (possible losses include all biotic and abiotic losses)

sample name	degr. day	PPO ISTD corrected	% of original concentration
B-22-0	0	223,895	**100**
B-22-4	4	137,304	**61**
B-22-9	9	66	**0**
B-22-14	14	308	**0**
B-22-21	21	45	**0**
B-22-29	29	212	**0**

1 % GS bioreactor (possible losses include photo-oxidation and volatilisation)

		BEE		DIPN 1		DIPN 2		PPO	
	degr. day	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration
B-23-0	0	3,292,269	**100**	3,286,734	**100**	3,735,846	**100**	208,458	**100**
B-23-4	4	2,657,240	**81**	3,065,311	**93**	3,629,146	**97**	120,009	**58**
B-23-9	9	386,990	**12**	2,705,250	**82**	3,160,348	**85**	65	**0**
B-23-14	14	322	**0**	3,391,012	**103**	3,965,206	**106**	87	**0**
B-23-21	21	388	**0**	3,423,486	**104**	4,082,077	**109**	132	**0**
B-23-29	29	1,270	**0**	3,321,734	**101**	3,838,969	**103**	80	**0**

1 % GS bioreactor (possible losses include those due to airborne contamination, photo-oxidation and volatilisation)

		BEE		DIPN 1		DIPN 2		PPO	
	degr. day	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration
B-24-0	0	2,995,359	**100**	3,706,186	**100**	4,344,158	**100**	240,973	**100**
B-24-4	4	1,347,384	**45**	3,633,202	**98**	4,260,851	**98**	82,574	**34**
B-24-9	9	227	**0**	3,778,007	**102**	4,450,452	**102**	1,624	**1**
B-24-14	14	452	**0**	3,661,510	**99**	4,295,027	**99**	215	**0**
B-24-21	21	326	**0**	3,452,014	**93**	4,082,082	**94**	166	**0**
B-24-29	29	3,046	**0**	2,607,506	**70**	3,079,520	**71**	201	**0**

1 % GS bioreactor (possible losses include all abiotic losses)

		BEE		DIPN 1		DIPN 2		PPO	
	degr. day	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration
B-25-0	0	3,446,343	**100**	3,812,096	**100**	4,459,616	**100**	264,772	**100**
B-25-4	4	252	**0**	4,673,576	**123**	5,624,925	**126**	178,031	**67**
B-25-9	9	421	**0**	3,697,589	**97**	4,349,634	**98**	70	**0**
B-25-14	14	248	**0**	4,309,275	**113**	5,138,358	**115**	900	**0**
B-25-21	21	114	**0**	3,762,555	**99**	4,430,885	**99**	74	**0**
B-25-29	29	405	**0**	3,193,670	**84**	3,724,936	**84**	1,167	**0**

1 % GS bioreactor (possible losses include all biotic and abiotic losses)

		BEE		DIPN 1		DIPN 2		PPO	
	degr. day	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration	ISTD corrected	% of original concentration
B-26-0	0	3,353,624	**100**	3,656,570	**100**	4,358,256	**100**	292,115	**100**
B-26-4	4	336	**0**	2,862,323	**78**	3,459,343	**79**	110,292	**38**
B-26-9	9	3,867	**0**	2,542,732	**70**	2,977,511	**68**	57,432	**20**
B-26-14	14	124	**0**	2,130,050	**58**	2,537,833	**58**	42	**0**
B-26-21	21	230	**0**	1,871,081	**51**	2,273,494	**52**	65	**0**
B-26-29	29	241	**0**	2,564,032	**70**	2,985,811	**69**	3,012	**1**

BATCH A DATA

sample	degr. day	BAB peak 1 ratioed to ISTD corrected peak 15	% of original concentration	BAB peak 2 ratioed to ISTD corrected peak 15	% of original concentration	BAB peak 3 ratioed to IS corrected pconcentration	% of original	BAB peak 4 ratioed to IS corrected pconcentration	% of original	BAB peak 7 ratioed to IS corrected p concentration	% of original	BAB peak 8 ratioed to IS corrected pconcentration	% of original	BAB peak 9 ratioed to I corrected p concentration	% of original	BAB peak 10 ratioed to IS corrected pconcentration	% of original
1 % ES control bioreactor (contains no ASS)																	
A-1-0	0	0.63	96	0.49	96	0.35	95	0.08	94	0.91	97	0.63	96	0.12	98	1.07	96
A-1-0	0	0.68	104	0.53	104	0.39	105	0.08	106	0.97	103	0.68	104	0.12	102	1.15	104
A-1-6	6	0.61	92	0.48	94	0.35	95	0.07	92	0.90	96	0.64	98	0.11	93	1.08	97
A-1-9	9	0.58	88	0.45	89	0.33	90	0.07	93	0.88	94	0.62	95	0.12	99	1.08	97
A-1-20	20	0.57	87	0.44	87	0.31	85	0.06	75	0.86	92	0.60	92	0.10	80	1.08	97
A-1-20	20	0.56	85	0.43	84	0.30	81	0.06	74	0.86	91	0.59	90	0.10	79	1.07	96
A-1-30	30	0.55	84	0.43	84	0.25	67	0.02	47	0.89	95	0.53	80	0.07	60	1.06	95
A-1-30	30	0.56	86	0.43	85	0.26	70	0.04	45	0.86	91	0.52	80	0.08	62	1.08	97
1 % ES bioreactor (rig 1)																	
A-9-0	0	0.65	100	0.50	100	0.35	100	0.06	100	0.93	100	0.66	100	0.13	100	1.06	100
A-9-2	2	0.65	100	0.50	99	0.37	105	0.08	137	0.91	98	0.66	101	0.12	94	1.07	101
A-9-6	6	0.62	96	0.48	96	0.35	100	0.08	133	0.90	96	0.64	98	0.11	90	1.09	103
A-9-9	9	0.58	90	0.45	90	0.32	91	0.06	107	0.87	94	0.61	93	0.08	67	1.08	102
A-9-9	9	0.61	94	0.47	94	0.34	97	0.07	114	0.89	96	0.63	96	0.09	68	1.09	103
A-9-13	13	0.59	91	0.45	89	0.25	71	0.00	6	0.85	91	0.45	68	0.01	7	1.08	102
A-9-16	16	0.57	88	0.41	82	0.17	48	0.01	14	0.81	87	0.31	47	0.01	4	1.07	101
A-9-23	23	0.50	77	0.31	62	0.05	13	0.01	11	0.68	73	0.15	23	0.00	3	1.07	101
A-9-30	30	0.48	75	0.25	49	0.02	7	0.01	12	0.60	65	0.11	17	0.00	3	1.04	99
A-9-49	49	0.35	54	0.06	11	0.01	1	0.01	10	0.29	31	0.05	8	0.00	3	1.03	97
A-9-73	73	0.21	33	0.02	4	0.00	1	0.00	7	0.13	14	0.02	3	0.00	3	0.89	84
1 % BAB control bioreactor (contians no ASS)																	
A-20-0	0	0.57	100	0.51	100	0.37	100	0.08	100	0.92	100	0.65	100	0.12	100	1.12	100
A-20-2	2	0.67	117	0.51	100	0.38	102	0.09	108	0.96	105	0.68	105	0.12	103	1.17	105
A-20-2	2	0.61	107	0.48	94	0.35	93	0.08	99	0.87	95	0.61	94	0.12	98	1.09	97
A-20-9	9	0.60	106	0.47	91	0.34	91	0.07	92	0.86	93	0.61	94	0.11	92	1.07	96
A-20-9	9	0.63	110	0.48	94	0.36	96	0.08	96	0.90	97	0.65	100	0.11	92	1.08	96
A-20-16	16	0.56	98	0.44	85	0.32	86	0.07	84	0.84	91	0.59	91	0.10	84	1.07	95
A-20-23	23	0.55	96	0.43	83	0.30	82	0.07	82	0.83	91	0.57	89	0.09	76	1.04	92
A-20-27	27	0.51	89	0.40	79	0.28	76	0.06	71	0.82	89	0.55	85	0.08	70	1.04	93
A-20-27	27	0.53	92	0.41	80	0.29	78	0.06	71	0.82	89	0.56	86	0.08	70	1.04	93
1 % BAB bioreactor (rig 1)																	
A-22-0	0	0.61	98	0.48	99	0.35	99	0.07	100	0.88	99	0.61	99	0.12	98	1.07	99
A-22-0	0	0.61	97	0.47	98	0.35	100	0.07	97	0.88	99	0.61	98	0.12	98	1.06	98
A-22-0	0	0.62	99	0.48	99	0.35	99	0.07	98	0.88	99	0.62	100	0.12	101	1.09	101
A-22-0	0	0.66	105	0.50	104	0.36	103	0.08	105	0.92	103	0.64	102	0.12	102	1.09	101
A-22-2	2	0.64	103	0.50	103	0.36	103	0.08	102	0.90	101	0.63	102	0.12	100	0.67	62
A-22-9	9	0.59	96	0.46	96	0.33	95	0.07	88	0.87	97	0.60	96	0.10	83	1.08	100
A-22-9	9	0.60	96	0.45	94	0.33	94	0.06	82	0.85	95	0.60	96	0.10	83	1.06	98
A-22-9	9	0.62	99	0.48	99	0.35	101	0.07	96	0.88	99	0.64	103	0.10	84	1.06	98
A-22-16	16	0.55	88	0.41	85	0.11	33	0.01	10	0.80	90	0.13	21	0.01	4	1.03	96
A-22-23	23	0.49	78	0.33	67	0.01	3	0.01	10	0.67	76	0.02	3	0.00	4	1.07	100
A-22-30	30	0.46	74	0.27	56	0.01	3	0.01	9	0.60	68	0.02	4	0.01	4	1.07	99
A-22-49	49	0.33	53	0.10	22	0.01	2	0.01	9	0.25	28	0.02	2	0.00	3	1.04	96
A-22-49	49	0.32	51	0.10	21	0.01	2	0.01	9	0.25	28	0.01	2	0.00	3	1.03	96
A-22-73	73	0.16	25	0.01	2	0.00	1	0.00	2	0.02	3	0.00	0	0.00	4	0.94	87
A-22-73	73	0.15	25	0.00	1	0.00	1	0.00	6	0.02	2	0.00	0	0.00	1	0.95	88
A-22-73	73	0.15	24	0.02	3	0.00	0	0.00	4	0.03	4	0.02	3	0.01	5	0.96	89

key: peak numb	retention time (min)	formula	chemical name
1	15.72	C16H26	1-butylhexyl benzene
2	15.85	C16H26	1-propylheptyl benzene
3	16.09	C16H26	1-ethyloctyl benzene
4	16.55	C16H26	1-methylnonyl benzene
5	16.86	C17H28	1-pentylhexyl benzene not resolved
6	16.9	C17H28	1-butylheptyl benzene not resolved
7	17.03	C17H28	1-propyloctyl benzene
8	17.29	C17H28	1-ethylnonyl benzene
9	17.74	C17H28	1-methyldecyl benzene
10	17.98	C18H30	1-pentylheptyl benzene
11	18.03	C18H30	1-butyloctyl benzene
12	18.18	C18H30	1-propylnonyl benzene
13	18.44	C18H30	1-ethyldecyl benzene
14	18.86	C18H30	1-methylundecyl benzene
15	19.05	C19H32	1-pentyloctyl benzene
16	19.12	C19H32	1-butylnonyl benzene
17	19.26	C19H32	1-propyldecyl benzene
18	19.52	C19H32	1-ethylundecyl benzene
19	19.94	C19H32	1-methyldodecyl benzene

sample	degr. day	BAB peak 11 ratioed to ISTD corrected peak 15	% of original concentration	BAB peak 12 ratioed to ISTD corrected peak 15	% of original concentration	BAB peak 13 ratioed to I corrected	% of original concentration	BAB peak 14 ratioed to I corrected	% of original concentration	BAB peak 15 assumes i change w.	% of original concentration	BAB peak 16 ratioed to I corrected	% of original concentration	BAB peak 17 ratioed to corrected	% of original concentration	BAB peak 18 ratioed to I corrected	% of original concentration	BAB peak 19 ratioed to I corrected	% of original concentration
1 % ES control bioreactor (contains no ASS)																			
A-1-0	0	1.01	98	0.77	99	0.54	100	0.10	99	1.00	100	0.62	99	0.45	96	0.29	96	0.05	95
A-1-0	0	1.05	102	0.78	101	0.54	100	0.10	101	1.00	100	0.63	101	0.47	102	0.32	104	0.06	105
A-1-6	6	1.02	99	0.77	100	0.54	101	0.09	93	1.00	100	0.62	99	0.46	100	0.32	106	0.05	97
A-1-9	9	1.02	99	0.77	99	0.48	90	0.10	97	1.00	100	0.63	100	0.46	100	0.31	102	0.05	99
A-1-20	20	1.02	99	0.76	97	0.52	96	0.08	83	1.00	100	0.62	99	0.45	99	0.30	99	0.04	83
A-1-20	20	1.00	97	0.75	96	0.52	96	0.08	84	1.00	100	0.62	99	0.45	98	0.30	99	0.04	83
A-1-30	30	1.02	99	0.76	98	0.49	91	0.08	75	1.00	100	0.63	100	0.44	96	0.28	94	0.04	80
A-1-30	30	1.05	101	0.75	97	0.49	92	0.08	78	1.00	100	0.62	98	0.44	97	0.30	98	0.04	79
1 % ES bioreactor (rig 1)																			
A-9-0	0	1.04	100	0.77	100	0.53	100	0.10	100	1.00	100	0.63	100	0.44	100	0.29	100	0.05	100
A-9-2	2	1.03	100	0.79	103	0.54	103	0.10	102	1.00	100	0.65	104	0.48	108	0.33	115	0.06	109
A-9-6	6	1.02	99	0.79	103	0.54	102	0.09	92	1.00	100	0.64	101	0.48	109	0.33	114	0.05	100
A-9-9	9	1.02	99	0.76	99	0.51	97	0.07	70	1.00	100	0.62	98	0.45	103	0.30	105	0.04	70
A-9-9	9	1.04	101	0.77	100	0.54	102	0.07	73	1.00	100	0.63	99	0.47	106	0.32	113	0.04	73
A-9-13	13	1.02	98	0.73	94	0.38	72	0.01	10	1.00	100	0.62	99	0.45	101	0.22	78	0.00	4
A-9-16	16	1.01	97	0.69	90	0.26	49	0.01	7	1.00	100	0.61	97	0.41	94	0.15	51	0.00	3
A-9-23	23	0.97	94	0.59	76	0.17	33	0.01	6	1.00	100	0.60	95	0.37	83	0.11	39	0.00	1
A-9-30	30	0.94	91	0.57	74	0.16	30	0.01	6	1.00	100	0.60	95	0.36	82	0.08	27	0.00	2
A-9-49	49	0.83	80	0.45	58	0.12	23	0.01	6	1.00	100	0.59	93	0.35	79	0.10	35	0.00	2
A-9-73	73	0.65	62	0.33	42	0.08	14	0.01	7	1.00	100	0.57	91	0.33	75	0.08	27	0.00	2
1 % BABs control bioreactor (contains no ASS)																			
A-20-0	0	1.08	100	0.77	100	0.54	100	0.10	100	1.00	100	0.64	100	0.46	100	0.31	100	0.05	100
A-20-2	2	1.12	104	0.83	107	0.57	105	0.11	108	1.00	100	0.68	107	0.49	106	0.32	103	0.06	109
A-20-2	2	1.05	97	0.76	98	0.52	97	0.10	101	1.00	100	0.63	99	0.46	100	0.47	151	0.07	124
A-20-9	9	1.00	92	0.75	96	0.51	95	0.09	94	1.00	100	0.63	98	0.46	100	0.31	98	0.05	96
A-20-9	9	1.04	97	0.78	101	0.54	101	0.10	97	1.00	100	0.65	103	0.49	107	0.33	106	0.06	103
A-20-16	16	1.01	94	0.74	96	0.51	94	0.08	80	1.00	100	0.62	98	0.45	98	0.29	94	0.04	84
A-20-23	23	1.01	94	0.73	94	0.49	91	0.08	75	1.00	100	0.63	99	0.45	98	0.29	94	0.04	77
A-20-27	27	1.02	94	0.73	94	0.48	89	0.05	52	1.00	100	0.62	98	0.44	96	0.28	90	0.04	68
A-20-27	27	1.01	93	0.73	94	0.48	89	0.07	69	1.00	100	0.62	97	0.44	96	0.28	90	0.04	71
1 % BABs bioreactor (rig 1)																			
A-22-0	0	1.05	101	0.75	99	0.52	99	0.10	99	1.00	100	0.64	100	0.45	99	0.29	100	0.05	96
	0	1.03	100	0.76	100	0.51	99	0.10	101	1.00	100	0.63	100	0.45	100	0.28	97	0.05	102
	0	1.02	99	0.76	100	0.52	100	0.10	99	1.00	100	0.64	101	0.45	100	0.30	102	0.05	99
	0	1.04	101	0.77	101	0.52	101	0.10	102	1.00	100	0.63	99	0.46	101	0.29	101	0.05	104
A-22-2	2	1.02	98	0.76	101	0.53	102	0.10	97	1.00	100	0.63	99	0.45	99	0.30	102	0.05	100
A-22-9	9	1.03	100	0.75	99	0.50	97	0.08	81	1.00	100	0.62	98	0.45	99	0.30	103	0.04	80
A-22-9	9	1.00	96	0.74	98	0.51	99	0.08	84	1.00	100	0.63	99	0.46	101	0.29	99	0.04	84
A-22-9	9	1.02	99	0.75	99	0.53	103	0.08	82	1.00	100	0.63	100	0.46	103	0.30	104	0.04	81
A-22-16	16	1.00	96	0.67	88	0.09	16	0.01	7	1.00	100	0.62	98	0.39	86	0.04	12	0.00	1
A-22-23	23	1.00	97	0.49	65	0.02	3	0.01	8	1.00	100	0.60	95	0.26	56	0.01	2	0.00	1
A-22-30	30	0.97	94	0.45	59	0.02	3	0.01	6	1.00	100	0.59	94	0.22	48	0.01	4	0.00	2
A-22-49	49	0.89	86	0.15	20	0.01	2	0.01	7	1.00	100	0.54	85	0.07	15	0.01	3	0.00	0
A-22-49	49	0.90	87	0.15	20	0.01	3	0.01	8	1.00	100	0.54	86	0.07	15	0.01	3	0.00	1
A-22-73	73	0.56	54	0.03	4	0.01	3	0.00	3	1.00	100	0.35	55	0.04	8	0.00	1	0.00	1
A-22-73	73	0.57	55	0.04	5	0.00	1	0.01	10	1.00	100	0.35	56	0.04	8	0.00	1	0.00	2
A-22-73	73	0.55	53	0.06	8	0.04	7	0.02	20	1.00	100	0.36	56	0.04	9	0.02	7	0.01	11

BATCH B DATA

sample name	degr. day	BAB peak 1 ratioed to ISTD corrected peak 15	% of original concentration	BAB peak 2 ratioed to ISTD corrected peak 15	% of original concentration	BAB peak 3 ratioed to ISTD corrected peak 15	% of original concentration	BAB peak 4 ratioed to ISTD corrected peak 15	% of original concentration
1 %ES bioreactor (possible losses include photo-oxidation and volatilisation)									
B-19-0 (1)	0	0.65	**101**	0.49	**99**	0.37	**102**	0.08	**102**
B-19-0 (2)	0	0.64	**100**	0.50	**101**	0.36	**100**	0.08	**97**
B-19-0 (3)	0	0.63	**99**	0.49	**100**	0.36	**99**	0.08	**100**
B-19-4	4	0.61	**95**	0.48	**97**	0.35	**96**	0.08	**99**
B-19-9	9	0.61	**96**	0.49	**99**	0.36	**98**	0.07	**93**
B-19-14	14	0.55	**86**	0.45	**90**	0.33	**89**	0.07	**87**
B-19-21	21	0.56	**88**	0.45	**91**	0.33	**91**	0.07	**84**
B-19-29	29	0.53	**83**	0.44	**88**	0.32	**87**	0.06	**75**
1 %ES bioreactor (possible losses include those due to airborne contamination, photo-oxidation and volatilisation)									
B-20-0	0	0.63	**100**	0.49	**100**	0.36	**100**	0.08	**100**
B-20-4	4	0.61	**97**	0.49	**101**	0.36	**99**	0.08	**100**
B-20-9	9	0.60	**94**	0.47	**98**	0.36	**98**	0.08	**99**
B-20-14	14	0.55	**88**	0.45	**93**	0.33	**91**	0.07	**89**
B-20-21	21	0.53	**85**	0.43	**88**	0.33	**90**	0.07	**93**
B-20-29	29	0.52	**83**	0.41	**84**	0.31	**85**	0.07	**90**
1 %ES bioreactor (possible losses include all abiotic losses)									
B-21-0	0	0.65	**100**	0.50	**100**	0.38	**100**	0.08	**100**
B-21-4	4	0.59	**92**	0.49	**98**	0.35	**93**	0.08	**90**
B-21-9	9	0.62	**96**	0.50	**99**	0.36	**96**	0.08	**95**
B-21-14	14	0.56	**87**	0.44	**88**	0.33	**87**	0.05	**61**
B-21-21	21	0.58	**89**	0.46	**92**	0.33	**88**	0.03	**33**
B-21-29	29	0.56	**87**	0.44	**89**	0.29	**78**	0.01	**12**
1 %ES bioreactor (possible losses include all biotic and abiotic losses)									
B-22-0	0	0.64	**100**	0.49	**100**	0.36	**100**	0.08	**100**
B-22-4	4	0.62	**97**	0.48	**97**	0.36	**98**	0.07	**83**
B-22-9	9	0.60	**95**	0.48	**97**	0.35	**98**	0.07	**88**
B-22-14	14	0.59	**92**	0.45	**91**	0.27	**75**	0.01	**17**
B-22-21	21	0.58	**91**	0.43	**87**	0.22	**62**	0.01	**8**
B-22-29	29	0.56	**87**	0.38	**77**	0.09	**26**	0.01	**10**

sample name	degr. day	BAB peak 11 ratioed to ISTD corrected peak 15	% of original concentration	BAB peak 12 ratioed to ISTD corrected peak 15	% of original concentration	BAB peak 13 ratioed to ISTD corrected peak 1	% of original concentration	BAB peak 14 ratioed to ISTD corrected peak 15	% of original concentration
1 %ES bioreactor (possible losses include photo-oxidation and volatilisation)									
B-19-0 (1)	0	1.06	**102**	0.80	**103**	0.56	**102**	0.10	**101**
B-19-0 (2)	0	1.03	**99**	0.77	**99**	0.55	**100**	0.10	**101**
B-19-0 (3)	0	1.03	**99**	0.77	**98**	0.54	**98**	0.10	**98**
B-19-4	4	1.07	**103**	0.80	**102**	0.55	**100**	0.10	**96**
B-19-9	9	1.05	**101**	0.78	**100**	0.54	**98**	0.10	**99**
B-19-14	14	1.06	**102**	0.78	**99**	0.54	**98**	0.09	**91**
B-19-21	21	1.05	**101**	0.77	**99**	0.54	**99**	0.08	**84**
B-19-29	29	1.03	**99**	0.77	**98**	0.53	**96**	0.07	**70**
1 %ES bioreactor (possible losses include those due to airborne contamination, photo-oxidation and volatilisation)									
B-20-0	0	1.04	**100**	0.78	**100**	0.55	**100**	0.10	**100**
B-20-4	4	1.07	**103**	0.78	**100**	0.56	**102**	0.10	**95**
B-20-9	9	1.04	**100**	0.78	**99**	0.56	**102**	0.10	**99**
B-20-14	14	1.03	**99**	0.77	**98**	0.53	**97**	0.09	**92**
B-20-21	21	1.06	**102**	0.77	**99**	0.55	**100**	0.10	**97**
B-20-29	29	1.01	**97**	0.75	**95**	0.54	**98**	0.10	**92**
1 %ES bioreactor (possible losses include all abiotic losses)									
B-21-0	0	1.04	**100**	0.78	**100**	0.56	**100**	0.10	**100**
B-21-4	4	1.07	**102**	0.79	**101**	0.54	**97**	0.10	**93**
B-21-9	9	1.05	**101**	0.80	**103**	0.56	**100**	0.10	**98**
B-21-14	14	1.05	**100**	0.77	**99**	0.54	**97**	0.09	**88**
B-21-21	21	1.06	**102**	0.79	**101**	0.53	**95**	0.05	**45**
B-21-29	29	1.03	**98**	0.78	**100**	0.49	**88**	0.03	**31**
1 %ES bioreactor (possible losses include all biotic and abiotic losses)									
B-22-0	0	1.05	**100**	0.79	**100**	0.54	**100**	0.10	**100**
B-22-4	4	1.05	**100**	0.79	**101**	0.55	**101**	0.09	**96**
B-22-9	9	1.05	**100**	0.77	**99**	0.54	**99**	0.08	**86**
B-22-14	14	1.07	**102**	0.73	**93**	0.43	**80**	0.03	**33**
B-22-21	21	1.03	**98**	0.72	**91**	0.36	**66**	0.01	**10**
B-22-29	29	0.99	**94**	0.64	**82**	0.28	**52**	0.01	**12**

BAB peak 7 ratioed to ISTD corrected peak 15	% of original concentration	BAB peak 8 ratioed to ISTD corrected peak 15	% of original concentration	BAB peak 9 ratioed to ISTD corrected peak 15	% of original concentration	BAB peak 10 ratioed to ISTD corrected peak 15	% of original concentration
0.90	**100**	0.66	**101**	0.12	**100**	1.09	**102**
0.90	**100**	0.65	**100**	0.12	**101**	1.07	**100**
0.90	**100**	0.65	**99**	0.11	**99**	1.06	**99**
0.91	**101**	0.64	**98**	0.11	**97**	1.10	**102**
0.93	**103**	0.67	**102**	0.11	**96**	1.10	**102**
0.86	**96**	0.63	**96**	0.10	**90**	1.06	**99**
0.91	**101**	0.63	**97**	0.09	**82**	1.08	**101**
0.86	**96**	0.62	**94**	0.08	**67**	1.06	**99**
0.89	**100**	0.65	**100**	0.11	**100**	1.07	**100**
0.91	**102**	0.66	**102**	0.12	**104**	1.09	**102**
0.91	**102**	0.65	**100**	0.12	**102**	1.06	**100**
0.86	**97**	0.63	**97**	0.11	**96**	1.06	**100**
0.87	**97**	0.64	**98**	0.11	**100**	1.05	**98**
0.85	**95**	0.62	**95**	0.11	**96**	1.06	**99**
0.91	**100**	0.66	**100**	0.12	**100**	1.11	**100**
0.93	**103**	0.64	**97**	0.11	**97**	1.08	**98**
0.93	**102**	0.67	**101**	0.12	**102**	1.08	**98**
0.87	**96**	0.63	**95**	0.11	**91**	1.06	**96**
0.92	**101**	0.62	**94**	0.04	**39**	1.08	**98**
0.88	**97**	0.57	**87**	0.03	**25**	1.06	**96**
0.91	**100**	0.65	**100**	0.12	**100**	1.10	**100**
0.91	**101**	0.65	**99**	0.11	**100**	1.10	**100**
0.90	**99**	0.65	**100**	0.10	**86**	1.06	**97**
0.87	**96**	0.51	**78**	0.04	**31**	1.09	**99**
0.85	**94**	0.45	**68**	0.01	**11**	1.11	**101**
0.75	**83**	0.28	**42**	0.01	**6**	1.06	**97**

BAB peak 15 assumes no change w. time	% of original concentration	BAB peak 16 ratioed to ISTD corrected peak 15	% of original concentration	BAB peak 17 ratioed to ISTD corrected peak 1:	% of original concentration	BAB peak 18 ratioed to ISTD corrected peak 1!	% of origir concentra	BAB peak 19 ratioed to ISTD corrected peak 15	% of original concentration
1.00		0.65	**101**	0.48	**101**	0.33	**100**	0.06	**102**
1.00		0.64	**99**	0.47	**99**	0.33	**100**	0.06	**99**
1.00		0.64	**100**	0.47	**99**	0.33	**99**	0.06	**99**
1.00		0.64	**100**	0.47	**100**	0.32	**96**	0.05	**97**
1.00		0.65	**101**	0.47	**100**	0.32	**97**	0.06	**98**
1.00		0.64	**100**	0.47	**99**	0.32	**95**	0.05	**89**
1.00		0.65	**101**	0.46	**98**	0.31	**95**	0.04	**74**
1.00		0.63	**98**	0.47	**100**	0.32	**98**	0.04	**68**
1.00		0.64	**100**	0.48	**100**	0.33	**100**	0.06	**100**
1.00		0.64	**100**	0.48	**100**	0.32	**98**	0.06	**96**
1.00		0.64	**100**	0.47	**98**	0.33	**99**	0.05	**91**
1.00		0.64	**100**	0.46	**97**	0.31	**94**	0.05	**86**
1.00		0.64	**99**	0.47	**99**	0.32	**98**	0.06	**96**
1.00		0.63	**97**	0.47	**98**	0.33	**100**	0.05	**93**
1.00		0.66	**100**	0.48	**100**	0.33	**100**	0.06	**100**
1.00		0.65	**99**	0.47	**98**	0.31	**95**	0.05	**94**
1.00		0.66	**100**	0.47	**97**	0.32	**98**	0.06	**98**
1.00		0.63	**96**	0.46	**97**	0.24	**74**	0.05	**92**
1.00		0.64	**97**	0.47	**98**	0.31	**93**	0.02	**40**
1.00		0.66	**101**	0.47	**99**	0.30	**91**	0.02	**28**
1.00		0.64	**100**	0.48	**100**	0.32	**100**	0.06	**100**
1.00		0.65	**102**	0.47	**99**	0.33	**103**	0.06	**99**
1.00		0.62	**98**	0.46	**98**	0.31	**97**	0.05	**81**
1.00		0.62	**98**	0.44	**92**	0.25	**78**	0.01	**21**
1.00		0.63	**99**	0.42	**87**	0.20	**63**	0.00	**5**
1.00		0.61	**96**	0.39	**82**	0.17	**52**	0.00	**3**

Set up and maintenanace costs	initial number	life span of equipment (years)	total number required over plant life-span	cost (£) per unit	total cost (£) over plant life-span
vial crusher	1	15.0	1	33,000	33,000
bioreactor	17	7.5	34	500	17,000
vessels for storage of ancillary liquids (6 per bioreactor)	102	7.5	204	50	10,200
tubing to gas traps	17	5.0	51	100	5,100
tubing from and to reservoirs	17	1.0	255	10	2,550
gas trap (2 per bioreactor)	34	5.0	102	50	5,100
air lift pump	17	5.0	51	50	2,550
other pumps (6 per bioreactor)	102	5.0	306	100	30,600
compressor	2	5.0	6	500	3,000
temperature probe	17	15.0	17	10	170
pH probe	17	5.0	51	50	2,550
dissolved oxygen probe	17	5.0	51	50	2,550
aeration sensor	17	5.0	51	100	5,100
level sensors (7 per bioreactor)	119	5.0	357	30	10,710
PC	1	15.0	1	700	700
data logger	1	15.0	1	2,000	2,000
BOD analysis kit	1	7.5	2	1,600	3,200
UV lighting kit	1	15.0	1	300	300
Mixer	17	7.5	34	200	6,800
				Total cost (£)	**143,180.00**

life expectancy of plant (years)	15
degradation time required (days)	21
additional time to fill, settle and empty bioreactors (days)	4
operating time per year (days)	300
number of runs annually per bioreactor	12.0
batch run time (days)	25
total vol. LSC used in UK annually (L)	80,000
total annual disposal by this process (L)	10,000
total batch run time (days)	25
Theoretical LSC volume per group of bioreactor runs (L)	833.3
LSC loading capacity	0.05
total bioreactor volume required (L)	16666.7
rounded up bioreactor volume required (L)	17000
Actual LSC volume per group of bioreactors (L)	850
number of bioreactors at 1m3 each (1000 L)	17
total number of batches per year	204
total volume LSC per bioreactor (L)	50
cost of electricity - commercial (£/kWh)	0.044
assumes no electrciity price increases	

blue = operational variable
red = estimate

Note : the waste is typically about 50 % LSC only
all calculations are for costs per L of LSC, with loadings being given as % of total consisting of LSC
All calculations are for a bioreactor size of 1,000 L

Conditions used

temperature	ambient
aeration rate (L/min) - in 1 m3 bioreactor	200
nutrient medium	standard

Commercial viability study

Commercial viability study

Running costs

	details		costs per unit (£)	average cost per bioreactor run (£)	total running costs over plant life-span (£)	annual running costs (£)
rental of space (incl. Storage)	per year	4000		19.61	60,000.00	4000.00
standard utilities	total costs per year	400		1.96	6,000.00	400.00
additional electricity - compressor	wattage (kW)	2.1		7.94	24,282.72	1618.85
additional electricity - air lift pump	wattage (kW)	0.105		3.37	10,320.16	688.01
additional electricity - mixer	wattage (kW)	1.0		22.18	67,858.56	4523.90
additional electricity - other pumps	wattage (kW)	0.105		2.66	8,143.03	542.87
additional electricity - UV-lighting kit	wattage (kW)	0.105		0.01	39.92	2.66
sample vials	total per run	5	0.07	0.35	1,071.00	71.40
BOD analysis	total per run	5	0.15	0.75	2,295.00	153.00
independent GC-MS analysis	total per run	2	20.00	40.00	122,400.00	8160.00
NaOH for gas trap	total per run (£)			3.00	9,180.00	612.00
nutrients	total per run (£)			20.00	61,200.00	4080.00
disposal costs - liquid and radioactive NaOH	charge per year (£)	3,500		17.16	52,500.00	3500.00
additional lab costs (miscellaneous)	charge per run (£)			3.00	9,180.00	612.00
			Total (£)	141.98	434,470.38	**28,964.69**

Additional costs

	cost	frequency (years)	total cost (£)	annual costs
Environmental impact assessment	7,000.00	one off	7,000.00	466.67
licence fees (£)	2,000.00	one off	2,000.00	133.33
Inspection/monitoring costs (£)	1,200.00	once/year	18,000.00	1,200.00
		Total	27,000.00	**1,800.00**

Total costs

	for plant lifespan (£)	per bioreactor run	per litre of LSC (£)
set up and maintenance costs	143,180.00	46.79	0.936
running costs	434,470.38	141.98	2.840
additional costs	27,000.00	8.82	0.176
Grand total (£)	604,650.38	197.60	**3.95**
total volume LSC processed per bioreactor (L)	50		

these costs exclude labour (maintenance) and collection/delivery costs (mileage and driver)

notes:-
the process will be an enclosed system
down time (weeks) 9.3
this includes time for adaptation of the biomass (every 8th batch for three weeks with a LSC loading of 5 %)

	per 60 L bin containing ~10 L LSC waste	per L of LSC waste	per L of LSC assuming waste is 50 % LSC
cost of incineration (includes collection)	£25-125	£ 2.50-12.50	£ 5.00 - 25.00

the remainder of the bin contents consists of principally of sample vials and caps

Glossary of terms

'end-of-pipe' treatment – clean-up treatment conducted at the end of a process

'sum-of-squares' error method – calculation of cumulative error by squaring the individual errors involved and adding them together

'whole-effluent' approach – tests carried out on the effluent in its entirety rather than on its individual constituents

2,5-diphenyl oxazole (PPO) – primary fluor present in both Gold Star™ and EcoSafe™

Abiotic losses – losses incurred by non-biological means e.g. evaporation

Absorbance – the logarithm of the ratio of the intensity of light incident on a sample to that transmitted by it. This is usually directly proportional to the concentration of the absorbing substance in the solution (Larousse, 1995)

Acclimation – to adapt or become accustomed to a new environment or conditions

Actinomyces – an order of bacteria producing a fine mycelium and sometimes arthrospores. Some members are pathogenic in animals and plants, and some produce antibiotics (Larousse, 1995)

Activated sludge – a mass of organisms consisting primarily of bacteria and protozoa. The biomass is combined with the sewage effluent from the primary filtration treatment. The mixture is then agitated and aerated for several hours, during which much of the soluble organic matter in the sewage is oxidise or assimilated by the microflora resulting in a marked decrease in the biological oxygen demand, and an increase in the activated sludge mass due to microbial growth and multiplication

Acute dose – fatal dose of radiation

Adsorption – accumulation of a substance on a solid surface, forming a film

Agar – a gelatinous carbohydrate obtained from seaweed, used as a culture medium for microorganisms, and a thickening agent (Collins Concise Dictionary, 1999)

Agar plating – the use of solidified agar jelly to cultivate microorganisms in Petri-dishes

Alicyclic ring – ring of carbon atoms having essentially aliphatic properties (Collins Concise Dictionary, 1999)

Alkyl chain – organic carbon-hydrogen chain containing the monovalent group C_nH_{2n+1}

Amphoteric – a compound partaking in both acidic and basic characteristics (Oxford English Dictionary Online, 2001)

Ångström - unit of wavelength for electromagnetic radiation covering visible light and X-rays. $1 Å = 10^{-10} m$

Anion – a negatively charged ion

Aromatic ring – unsaturated ringed organic compound

Autoclaving – to sterilise something by means of steam under pressure

Bacteria – a large group of typically unicellular microorganisms

Bactericide – a substance able to destroy bacteria

Bandpass region – region of a frequency range where ions are able to pass on to the detector in a mass spectrometric system. Ions passing through depend on their mass-to-charge ratio corresponding to the frequency range

Basal salt medium – basic nutrient medium used to culture microorganisms

Base peak – the most intense peak in a mass spectrum

Batch reactor – reactor which operates with a single content change per cycle, as opposed to a continuous reactor in which there is continuous flow of content through the system

Becquerel (Bq) – the standard unit of activity of a radioactive source

Binary fission – division of the nucleus into two daughter nuclei, followed by similar division of the cell body (Larousse, 1995)

Bioaccumulation – process which causes an increased chemical concentration in an organism as compared to its environment, due to an uptake by all exposure routes, including dietary and dermal absorption, and respiration (Mackay & Fraser, 2000)

Bioaugmentation – the artificial increase of microbial concentrations to enhance degradation rates, i.e. the introduction of a pre-adapted culture

Biodegradation – 'a natural process whereby bacteria or other microorganisms alter and break down organic molecules into other substances, eventually producing fatty acids and carbon dioxide' (Hoff, 1993)

Biokinetic – the kinetics of biologically mediated reactions

Biological oxygen demand – the amount of dissolved oxygen needed for the microbial oxidation of biodegradable matter. The BOD_5 test measure the oxygen (mg) consumed per litre of liquid, or a known dilution thereof, in five days at 20 °C

Biomass – the total weight of the organic substance or organisms in a given area

Bioreactor – a fermentor or other apparatus used for bioconversions

Bioremediation – 'the acceleration of the biodegradation process through addition of nutrients or other materials to contaminated environments or through manipulation of the contaminated media using techniques such as aeration or temperature control' (Hoff, 1993)

Biostimulation – optimising conditions for biodegradation by modifying environmental conditions like temperature or nutrient content

Biotic losses – losses by biological means e.g. biodegradation

Bis-methylstyrl benzene (bis-MSB) – secondary fluor present in Gold Star™ and EcoSafe™

Branched alkyl benzene (BAB) – primary solvent present in EcoSafe™

Butoxyethoxy ethanol (BEE) – co-solvent present in Gold Star™ and EcoSafe™

Capsular extracellular protein – protein forming a capsule outside the cell

Carbonise – to turn or be turned into carbon as a result of heating, chemical treatment etc. (Collins Concise Dictionary, 1999)

Carcinogenic – substance that has the capability to cause cancer

Carrier gas – gas in a gas chromatograph system that carries each compound in turn to the detector as they partition out of the solid phase with increasing temperature

Cation – positively charged ion

Chemical oxygen demand – a measure of the amount of potassium dichromate needed to oxidise reducing material in a liquid sample. It is expresses as the concentration of oxygen gas (ppm) chemically equivalent to the amount of dichromate consumed. It is generally higher than the biological oxygen demand, as some materials, such as cellulose, will react with dichromate but not with oxygen under biological conditions

Chromatography – 'a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary while the other moves in a definite direction' IUPAC's definition (Ettre, 1993)

Climate Change Levy – legislation that will be introduced in April 2001 to tax business use of energy and result in a reduction in UK greenhouse gas emissions

Colony forming units – microbial cells capable of developing into a new colony when subjected to suitable culture conditions

Cometabolism – 'where one organism that is growing on a particular substrate also oxidises a second substrate that it is unable to assimilate or use as a carbon and energy source. The oxidation products of the second substrate are then used by other bacteria in the community' (Singleton, 1994)

Competent biomass – microbes possessing the genetic capability to metabolise a specific carbon source and utilise it for growth

Complete combustion – full oxidative combustion resulting in the formation of carbon dioxide

Continuously pumped system – a bioreactor system where fresh activated sludge is continuously pumped into the bioreactor whilst processed effluent is removed

Copepod – a minute marine or freshwater crustacean, and an important component of plankton (Collins Concise Dictionary, 1999)

Covalent bonding – the formation of chemical bonds where electrons are shared between atoms in a molecule

Critical micelle concentration – see micelle

Cross-contamination – contamination passing from one sample/analysis to the next

Cuvette – sample-holding vessel used in spectrophotometry, which is transparent to light, and can be made of a variety of materials including silicon and quartz

Cytochrome – proteins of the electron transfer chain that can carry electrons due to the presence in their structures of haem prosthetic groups (Larousse, 1995)

D_8-naphthalene – internal standard used for GC-MS analysis

Death phase – phase of microbial population cycle where death exceeds regeneration

Desorption – reverse process to adsorption

Detection limit – the smallest sample quantity that yields a signal that can be distinguished from the background noise (de Hoffmann *et al,* 1996)

Detector saturation – saturation of the detector such that response is no longer linearly related to compound concentration. This results in non-Gaussian shaped peaks in mass spectrometry

Detergent – cleaning agents (solvents, or mixtures thereof, sulphonated oils, abrasives etc.) for removing dirt, paint etc. (Larousse, 1995)

Dichloromethane – solvent used for iquid-liquid extraction purposes

Di-isopropyl naphthalene (DIPN) – primary solvent present in Gold Star™

Dioxins – a family of toxic and persistent chlorinated aromatic hydrocarbons. They are lipophilic and are found as trace compounds in the food chain (Larousse, 1995)

Dipole-dipole interactions – interaction of a pair of molecules or atoms with non-coincident equal and opposite electric charges or magnetic poles

Direct injection – injection of a sample into a GC system via an inlet sleeve, where the sample can volatilise and contaminants can be trapped

Drift monitor – a standard or sample injected into the GC system on a regular basis to determine any retention time changes

Dynode – intermediate electrode (between cathode and final anode) in photomultiplier tube. Dynode electrons are those that emit secondary electrons and provide amplification (Larousse, 1995)

EcoSafe™ – liquid scintillant cocktail used in this study

Effluent – the liquid or gaseous waste from a chemical or other plant

Electron impact ionisation (EI) – ionisation of a gaseous sample by a beam of electrons most often accelerated by a potential of about 70 eV (de Hoffmann *et al,* 1996)

Elution time – time following injection of a sample at which it is detected e.g. in a GC-MS system

Emulsification – the formation of an emulsion

Emulsifier – a substance which makes an emulsion more stable by reducing the surface tension or protecting the droplets with a film (Grant, 1969)

Emulsion – a fluid consisting of a microscopically heterogeneous mixture of two normally immiscible liquid phases, in which one liquid forms minute droplets suspended in the other (Grant, 1969)

Environmental impact assessment (EIA) – also known as an environmental impact statement (EIS). A detailed analysis required of any operation undertaking a major project, of its effects on the environment

Epifluorescence microscopy – optical microscopy in which fluorescence is induced as a result of irradiation directed on the object from the same side as that from which it is viewed

Ether cleavage – the splitting of an ether molecule. The ether molecule being one where two identical, or different alkyl groups are united to an oxygen atom, forming a homologous series $C_nH_{2n+2}O$ (Larousse, 1995)

Ethoxylate – a grouping where oxygen substitution has occurred onto the ethoxy group ($O.C_3H_5$)

Excalibur™ – GC-MS software used in this study

Excitation energy – energy required to raise an atom or nucleus above the ground state

Excitation wavelength – wavelength projected at a sample

ex-situ – away from the original site, e.g. remediation of ground pollution in bioreactors

Fenton's reagent – an oxidising agent

First order rate equation – equation used to describe compound concentration in a first-order reaction, which is where the rate of the reaction is proportional to the concentration of a single reactant

Flammability – capacity of a substance to be inflammable, readily combustible

Flash point – the temperature at which a material gives off a vapour which will ignite on exposure to a flame

Floc – finely divided precipitates

Flue gas – the gaseous products of combustion from a furnace or incinerator, consisting chiefly of carbon dioxide, carbon monoxide, oxygen gas, nitrogen gas and water vapour

Fluor – a substance that emits light on absorption of radiation. Emission of light occurs as electrons excited to an elevated electron orbital return to their ground state. The light emitted is of a longer wavelength than that of the excitation radiation, resulting in a wavelength shift

Fluorescence – emission of radiation, generally light, from a material during illumination by radiation of usually higher frequency, or from the impact of electrons (Larousse, 1995)

Free radical – atom or group of atoms containing at least one unpaired electron, very unstable

Gold Star™ – liquid scintillant cocktail used in this study

Gram staining – a technique used to classify bacteria by staining them with a violet iodine solution (Collins Concise Dictionary, 1999)

Graticule – a transparent calibrated cell bearing a grid, cross-wire, or scale. Used in conjunction with a microscope to maesure or count objects in the field of view. Often the graticule is of a known volume and therefore can be used to quantify particles per unit volume

Grating – an arrangement of alternate reflecting and non-reflecting elements, which, through diffraction of the incident radiation, analyses this into its frequency spectrum (Larousse, 1995)

Half-life – time in which half of the atoms of a given quantity of radioactive nuclide undergo at least one disintegration (Larousse, 1995)

Halogenation – the introduction of halogen atoms into an organic molecule by substitution or addition (Larousse, 1995)

Headspace – the space between the surface of the liquid contents of the bioreactor and the top of the vessel

Homologous series – a series of organic compounds each member of which differs from the next by the insertion of a $-CH_2-$ group in the molecule. Such a series may be represented by a general formula and shows a gradual and regular change of properties with increasing molecular weight (Larousse, 1995)

Hot needle injection – injection of a sample into a hot injector port

Humic acids – complex organic acids occurring in the soil and in bituminous substances formed by the decomposition of dead vegetable matter

Hydrogen bond – a weak inter- or intramolecular force resulting from the interaction of a hydrogen atom bonding with an electronegative atom with a lone pair of electrons e.g. oxygen or nitrogen. Hydrogen bonding is important in associated liquids, particularly water (Larousse, 1995)

Hydrophilic – the capacity to be attracted to water molecules

Hydrophobic – the opposite of hydrophilic, therefore repelled by water molecules

Injector liner – glass tube, placed in the gas chromatographic injector, into which the syringe needle is placed to inject the sample. This can be packed with an inert material to trap some of the interferents in the sample

Inoculum – the biomass source used

in-situ – at the original site, e.g. remediation of ground pollution in by direct addition of microbes and nutrients to the beach for the remediation of oil pollution

Internal standard – substance added to a sample to assess procedural or analytical losses, which can then be used as a correction factor for the sample data

Internally conserved standard – substance only minimally affected by biotic or abiotic losses, and whose concentration is therefore conserved over very long time periods

Ion chromatography – a chromatographic method used to measure ions in solution. Ideal for quantification of nutrient ions like nitrates and phosphates

Ionisation – a process that yields an ion from an atom or neutral molecule (de Hoffmann *et al*, 1996)

Ionisation energy – the energy required to enable ionisation

Isomerism – the existence of more than one substance having a given molecular composition and relative molecular mass, but differing in constitution or structure. The compounds themselves are called isomers (Larousse, 1995)

Isothermal injection – injection of a sample into a gas chromatogram at a set temperature, with no further changes in injector temperature

Isotope – one of a set of chemically identical species of atom which have the same atomic number but different mass numbers. A few elements have only one natural isotope, but all elements have artificially produced radioisotopes (Larousse, 1995)

Isozyme – electrophoretically distinct forms of an enzyme with identical activities, usually coded by different genes (Larousse, 1995)

Kinetic energy – energy arising from motion

Kreb's cycle – also known as the TCA cycle, or tricarboxylic acid cycle. The biochemical pathway, whereby, in the presence of oxygen, pyruvic acid formed by glycolysis is broken down to form carbon dioxide and water, with the release of large amounts of energy in the form of ATP. It takes place in the mitochondria (Larousse, 1995)

Lag period/phase – the period during which whilst a cell acclimatises to the conditions and a second layer of cells is gradually formed

Lag time – the time taken for the lag phase to occur

Latent time – time between radiation and the onset of the effects e.g. development of a tumour, which may take from a few to tend of years, and is a function of age and tumour type

Limiting phase – this occurs when nutrient diffusion to the centre of the colony is limited, and/or there is a build up of toxic products, instigating a decrease in growth rate with further growth being limited to the peripheral zones of the colony, and a resulting linear increase in the diameter of the colony

Liquid chromatography – this is where a liquid is present as the carrier medium, as opposed to a gas in gas chromatography

Liquid-liquid extraction – the removal of a substance from a solution or a mixture of liquids by utilising its greater solubility in another liquid (Oxford University Press, 2001)

Liquid scintillant cocktail – a mixture of organic compounds including solvents, fluors and surfactants, used in the quantification of radionuclides

Liquid scintillant counting – the quantification by a liquid scintillation counter of small flashes of visible or ultraviolet light emitted by fluorescence in a scintillator/fluor when it is struck by a charged particle or high-energy photon

Low-level waste – those radioactive wastes which, because of their low activity, do not require shielding during normal handling or transport. The waste must contain less than 4 GBq of α activity and less than 12 GBq of β/γ activity per tonne

Lypophilicity – the tendency to be attracted to lipids in the tissue where that compound is then often absorbed and stored

Mass filter – a filter only allowing for ions with a specific mass-to-charge ratio to pass on to the mass spectrometric detector. Controlled by an upper and lower frequency limit

Mass spectrometer – an instrument that analyses ions according to their m/z ratio and which electrically measures the number of ions (de Hoffmann *et al*, 1996)

Mass spectrum – a record obtained with a mass spectrometer, in which ions from a sample material are represented as dispersed according to their mass-to-charge ratio (Oxford University Press, 2001)

Mass unit (u) – also known as a Dalton (Da). It is defined as $1/12^{th}$ of the mass of a ^{12}C atom $1\ u = 1\ Da = 1.660540 \times 10^{-27}$ kg (de Hoffmann *et al*, 1996)

Mass-to-charge ratio (m/z) – unit used to classify an ion, based on its molecular mass and ionic charge. Used for identification and separation purposes in mass spectrometry

Membrane bioreactor (MBR) – a modified batch reactor using of immobilised biomass, where the microorganisms are attached to a support by adsorption or covalent bonding

Metabolic capability – the capacity to metabolise a specific substrate

Metabolism – the sum-total of the chemical and physical changes constantly taking place in living matter (Larousse, 1995)

Metabolite – a substance involved in metabolism, being either synthesised during metabolism or taken in from the environment (Larousse, 1995)

Methanogensis – the production of methane

Micelle – colloidal aggregate of molecules formed in solution, especially soaps in water. Particles are often spherical with hydrophobic chains in the centre surrounded by hydrophilic groups. Only formed above a certain limit known as the critical micelle concentration (Larousse, 1995)

Microbial adaptation – a change in the microbial community resulting in an increased transformation rate of a compound as a result of prior exposure to that compound (Spain & van Veld, 1983). For further details see section 3.1.1

Microsome – the membranous pellet obtained by centrifugation of a cell homogenate after removal of the mitochondria and nuclei (Larousse, 1995)

Microtox® bioassay – a test for toxicity measuring the decrease in respiration, and subsequent light output, of a luminescent bacterium. It is a rapid tool for assessing toxicity of effluents, sediments, soils, sludges, ground water and surface water

Microwave assisted extraction – a method of solvent extraction involving a reaction vessel into which the sample and solvent are placed, which is subsequently placed in a microwave where a combination of heat and pressure aids extraction

Mineralisation – the complete breakdown of a chemical into its mineral constituents (Singleton, 1994)

Mixed liquor – the activated sludge type used in this study. A mixture of aerated wastewater and sludge biomass

Mobile phase – this refers to the carrier medium, which in the case of gas chromatography is the carrier gas like Helium or hydrogen

Molecular ion – ion produced by removing an electron from the sample molecule (de Hoffmann *et al,* 1996)

Mutagen – a substance which causes mutations

Natural selection – an evolutionary theory regarding the survival of the best adapted forms, with the inheritance of those characteristics wherein their fitness lies, and which arise as random variations due to mutations. First proposed by Charles Darwin, and is often referred to as the Darwinian Theory (Larousse, 1995)

Non-ionic detergent – series of detergents in which the molecules do not ionise in aqueous solution, unlike soap and sulphonated alkylates. Typical examples are detergents based on condensation products of long-chained glycols and octyl- or nonyl hydroxybenzenes (Larousse, 1995)

Nonylphenol ethoxylate (NPE) – emulsifier present in both Gold Star™ and EcoSafe™

Nutrient agar – an artificially prepared solution containing agar and all of the mineral substances and carbon sources required for general microbial growth. Used to make up nutrient agar plates for microbial cultivation

On-column injection – where the syringe needle passes straight into the end of the column to inject the sample and therefore no injector sleeve is involved. Only used for 'clean' samples at low concentrations

Optical density – absorbance is a measure of the optical density

Optiphase HiSafe™ – a liquid scintillant cocktail not used in this study

Orbital – a possible pattern of electron density in space which can be realised by two electrons at the most in an atom or molecule (Oxford University Press, 2001)

Oxidant – an oxidising agent; a substance that readily gives off oxygen (Oxford University Press, 2001)

Oxidation – the addition of oxygen to a compound. More generally, any reaction involving the loss of electrons from an atom, accompanied by reduction (Larousse, 1995)

Oxygenase enzyme – any enzyme which catalyses the incorporation of molecular oxygen into a substrate

Partition coefficient – the ratio of the equilibrium concentrations of a substance dissolved in two immiscible solvents (Larousse, 1995)

Percentage of the total intensity – abundance of an ion over the total abundance of all ions within a specified mass region

Photocathode – electrode from which electrons are emitted on the incidence of radiation in a photocell (Larousse, 1995)

Photomultiplier – photocell with a series of dynodes used to amplify emission current by electron multiplication, as used in a scintillation counter (Larousse, 1995)

Photon – a quantum of visible light or electromagnetic radiation of energy E=hv where h is Planck's constant and v is frequency (Larousse, 1995)

Photo-oxidation – also known as photochemical breakdown. A chemical breakdown as a result of radiation e.g. a light-catalysed reaction

Photosynthesis – the use of energy from light to drive chemical reactions e.g. reduction of carbon dioxide to carbohydrates coupled with the oxidation of water to oxygen gas or hydrogen sulphide to sulphur

Phthalate – a salt of phthalic acid, a common contaminant in mass spectra given off by injector septa

Physiosorption – the process of attachment of non-ionic substances such as polar water molecules to solid-phase surfaces

Polarity – a permanent property of a molecule which has an unsymmetrical electron distribution (Larousse, 1995)

Polyaromatic hydrocarbons – hydrocarbons consisting of two or more benzene rings. They constitute a group of priority pollutants, with some acting as mutagens and carcinogens

Precision – a reflection of reproducibility rather than accuracy which reflects the proximity to an expected value

Primary aliphatic chain – the shorter of the two alkane chains attached to the benzene ring in branched alkyl benzenes

Primordial – a term used to describe radionuclides thought to have been present near the time of the origin of the universe (Larousse, 1995)

Protozoa – a phylum of unicellular or acellular animals. Nutrition is holophylic, holozoic or saprophytic, reproduction is by fission or conjugation, locomotion by cilia, flagella or pseudopodia, and they are either free-living or parasitic (Larousse, 1995)

Pyrolysis – the decomposition of a substance by heat

Quadrupole (quad) – this consists of four parallel rods (quads) equally spaced around a central axis, controlling the ions passing to the detector by a mass filter

Quadrupole analyser – a mass filter that produces a quadrupolar field with a DC component and an AC component such that only ions with a specific m/z pass through the 'filter' (de Hoffmann *et al*, 1996)

Quantitative structure-biodegradability relationships (QSBR) – 5-46

Radioactive activity – this describes the rate at which transformations occur. The unit is a Becquerel (Bq), where 1 Bq = 1 nuclear disintegration per second

Radionuclide – any isotope of an element which is unstable and undergoes natural radioactive decay

Rate constant – coefficient relating the rate of a chemical reaction at a given temperature to the concentration of reactant (in a unimolecular reaction), or to the product of the concentrations of reactants (in a reaction of higher order) (Oxford University Press, 2001)

Recalcitrant – resistant to microbial attack for mineralisation

Recovery – Fraction of the original compound concentration that is still present at the end of a process e.g. liquid-liquid extraction

Relative intensity – ratio of the peak intensity to that of the base peak

Remediation – the act of remedying. A term often used to describe the clean up of pollution

Resolution – the act, process, or capability of making the separate peaks on a chromatogram distinguishable, particularly when elution times are very similar

Respirometer – a device which measures the rate of consumption of oxygen by a living or organic system (Oxford University Press, 2001)

Retention time (R_t) – as for elution time, the time following injection of a sample at which it is detected e.g. in a GC-MS system

Ring cleavage – the splitting up of an organic molecule ring into simpler molecules, usually by hydrolysis and mediated by an enzyme

Ring fission – as for ring cleavage, the splitting up of an organic molecule ring into simpler molecules, usually by hydrolysis and mediated by an enzyme

Ringers solution – an isotonic medium used in the dilution of microbial broth to ensure that the cell structure is not damaged by osmosis as a result of salt gradients across the cellular membrane

Salting-out – when an organic compound is driven out of aqueous solution by the addition of a salt (Oxford University Press, 2001)

Saturated alkane – alkane containing no double bonds

Scan time – counting time per mass in mass spectrometry

Scanning mass spectrometry – a mass spectrometer that scans across the selected mass-to-charge range rather than only detecting a single, or several selected ions

Scintillator – a substance capable of emitting flashes of light as a result of the impact of photons (Collins Concise Dictionary, 1999)

Selected ion monitoring (SIM) – conducted to increase the senisitvity of a mass spectrometric measurement by only detecting at specific mass-to-charge ratios to analyse selected ions only

Sensitivity – this is the ratio of the ionic current change to the sample flux change in the source. The analytical sensitivity is the smallest quantity of the sample compound yielding a definite signal to noise ratio, typically of 10:1 (de Hoffmann *et al,* 1996), also see detection limit

Sequencing batch reactor (SBR) – most standard bioreactor type, where aeration and separation are carried out sequentially in a single tank (Tchobanoglous & Burton, 1991)

Shake flask die-away test – standard test conducted by the US Environmental Protection Agency to assess the biodegradation of chemicals in natural surface water samples by means of the test compound being dissolved in water collected from a field site, with and without added natural sediment, and with and without sterilisation. Initial test compound concentrations typically are relatively low (micrograms per litre), analytical capabilities permitting. Loss of test compound with time is followed by an appropriate compound-specific analytical technique

Sievert (Sv) – used to denote either of two units of dose of ionising radiation

Signal-to-noise ratio – the ratio of the strength of a desired signal to that of unwanted noise interference (Oxford University Press, 1995)

Slime extracellular protein – protein forming a slime outside the cell

Solenoid valve – a valve actuated by the movement of a core into or out of a solenoid when an electric current is passed through the latter (adapted from Oxford University Press, 2001)

Solid-phase extraction (SPE) – a chromatographic technique used for sample preparation to remove interfering substances that may be present. This is done either by retaining the substance of interest and washing off the remaining compounds or by retaining the interfering substances and eluting the product of interest

Solubility – the extent to which one substance will dissolve in another. Usually expressed as the mass or quantity of substance that will dissolve in 1 L of water (Larousse, 1995)

Solvent blank – an aliquot of solvent injected into the system to determine contamination and carry-over

Solvent front – period at start of sample analysis by GC-MS where solvent ions pass through the detector

Sorption – a general term for the processes of adsorption, absorption, chemisorption and persorption (Larousse, 1995)

Soxhlet extraction – a method of continuous solvent extraction of a solid by means of heat and refluxing

Spent biomass – biomass that needs to be replaced, as it can no longer carry out its required function

Split event – a period of time whilst the split vent is open to allow for the injector to the purged of any remaining sample, ensure that there is no gradual sample transfer onto the column resulting in peak broadening and a loss of resolution

Split vent – the section of the gas chromatographic injector that can be opened by a solenoid valve to allow for a split event to occur

Split/splitless injector – a gas chromatographic injector type allowing for a split event

Stable concentration plateau – period of no change in analyte concentration

Stationary phase – the section of a chromatographic system out of which the analytes gradually elute, e.g. the column lining in gas chromatography

Substrate – reactant in a reaction catalysed by an enzyme, or surface or medium, on or in which an organism lives and from which it may derive nourishment (Larousse, 1995)

Supercritical fluid extraction – a technique in which CO_2 is used under extremely high pressure to separate a solution

Surfactant – an amphipathic molecule consisting of a hydrophilic polar head and a hydrophobic nonpolar tail. The term 'surfactant' is derived from SURFace ACTive AgeNT. Although surfactants can act as emulsifiers, not all emulsifiers are surfactants (Rouse *et al.*, 1994)

Synergistic – where the result of the combined action of two or more agents is greater than the sum of their separate, individual actions (Larousse, 1995)

Tandem mass spectrometry – where two or more mass spectrometers are arranged in tandem. Often used to obtain further structural information

Temperature programmable injector – a gas chromatographic injection system where a temperature programme can be used, allowing for the cold injection of the sample, followed by a ramping of the temperature to rapidly transfer the compounds into the column

Tertiary carbon atom – carbon atom linked to three other carbon atoms and one hydrogen atom, Generally less stable than secondary carbon atoms, and a weak point in polymer chains, making them more susceptible to degradation and oxidation (Larousse, 1995)

Testmix – a mixture of compounds supplied with a GC column used to test system performance like retention times and sensitivity

Thermally labile – prone to instability of form or nature as a result of heating

ThermoQuest/Finnigan Trace MS 2000™ – GC-MS system used in this study

Thomson (Th) – is the *m/z* unit. Where m/z is the mass-to-charge ratio with *m* in daltons and *z* as the charge (de Hoffmann *et al,* 1996)

Transfer line – section connecting the GC oven and MS through which the GC column passes prior to entering the ionisation chamber of the mass spectrometer

Troposphere – the lower part of the atmosphere, extending from the surface up to a height varying from 9 km at the poles to 17 km at the equator, in which the temperature decreases fairly regularly with height (Larousse, 1995)

Turbidity – the 'cloudiness' of a solution, usually as a result of suspended particulates

UV-fluorescence spectrometry – analytical technique using light of the ultra-violet wavelengths as the incident light beam shining onto a sample, and the detection of the fluorescence intensity emitted by the sample to quantify the fluorescing component of the sample. Often used for hydrocarbon quantification and identification

van der Waals forces – weak attractive forces between atoms or molecules which vary inversely as the sixth power of the interatomic or intermolecular distances, and are due to momentary dipoles cased by fluctuations in the electronic configuration of the atoms or molecules (Larousse, 1995)

Vaporisation – also known as volatilisation, the conversion of a liquid or solid into a vapour

Replica plating – technique used to transfer microbial colonies from one agar plate onto another, utilising a velvet sheet stretched over a frame that is gently pushed onto the agar plate with the existing cultures and then on to a new plate. Often used as a technique to maintain a microbial culture

Viable cell count – a technique to quantify the microbial cells that are capable of living and able to maintain a separate existence

Watercourse – a stream or body of water

Xenobiotic – a substance foreign to the body (Oxford University Press, 2001)

Zwitterionic – a hybrid structure derived from a number of ionic states. A substance can be neutral, acidic, basic or zwitterionic (Oxford University Press, 2001)

β- oxidation – an oxidation method common for fatty acid break down., specifically those with even number of carbon atoms. Occurs in the mitochondria under aerobic conditions, and involves a series of stages where hydrogen ions are removed from the fatty acid structure along with 2-carbon units, forming FADH and NADH

Abbreviations, acronyms and units

adsorp. (s)	sorptive losses
AES	alcohol ether sulphonate
airborne (a)	losses due to airborne contaminating microbes
APHA	American Public Health Association
AS	alcohol sulphonate
AS	activated sludge
ATP	adenosine triphosphate
BAB	branched alkyl benzene
BAB 1	branched alkyl benzene isomer 1
BEE	butoxyethoxy ethanol
Bis-MSB	bis-methylstyryl benzene
BOD	biological oxygen demand
BOD_5	biological oxygen demand (measured over 5 days)
CFU	colony forming units
COD	chemical oxygen demand
d_8	d_8-naphthalene
DCM	dichloromethane
DDT	dichloro diphenyl trichlorothane
DIPN	di-isopropyl naphthalene
DIPN 1	di-isopropyl naphthalene rig 1
EA	Environment Agency
EC	European Community
EIA	environmental impact assessment
EIS	environmental impact statement
ENDS	environmental data service
EO	ethoxylate
EPA	Environmental Protection Agency
eq.	equivalent concentration level to that present in liquid scintillant cocktails
ES	EcoSafe™
ES 1	EcoSafe™ rig 1
ES-MS	electrospray ionisation mass spectrometry
FT	Fourier Transform mass spectrometry
GC	gas chromatography
GC-MS	gas chromatography mass spectrometry
GS	Gold Star™
GS 1	GoldStar™ rig 1
HLW	high level waste
HM	Her Majesty's
HPLC	high pressure liquid chromatography
HSE	Health and Safety Executive
HTO	tritiated water
ICRP	International Commission on Radiological Protection
IPPC	Integrated Pollution Protection Control
LAB	linear alkyl benzene
LAS	linear alkylbenzene sulphonate
LLW	low level waste
LS	liquid scintillant
LS	liquid scintillant
MBR	membrane batch reactor
MLW	medium level waste
MoD	Ministry of Defence
MS	mass spectrometry
N/a	not applicable
NAD	nicotinamide adenine dinucleotide, a coenzyme I
NADH	reduced NAD
$NADH_2$	reduced NAD
NADP	nicotinamide adenine dinucleotide phosphate, a coenzyme II

NADPH	reduced NADP
$NADPH_2$	reduced NADP
NCIMB	National Collection of Industrial and Marine Bacteria
NPE	nonylphenol ethoxylate
NPE	nonylphenol ethoxylate
NRPB	National Radiological Protection Broad
OECD	Organisation for Economic Co-operation and Development
PAH	polyaromatic hydrocarbon
photo (p)	photo-oxidation losses
PPO	2,5-diphenyl oxazole
PPO 1	diphenyl oxazole rig 1
QSBR	quantitative structure-biodegradability relationship
SAS	secondary alkane sulphonate
SBR	sequencing batch reactor
SEPA	Scottish Environmental Protection Agency
SIM	selected ion monitoring
TIC	total ion current
ToF	Time-of-flight mass spectrometry
UV	ultra-violet
v/v	volume for volume
vol. (v)	volatilisation losses
WWTP	wastewater treatment plant
UV-FS	ultra violet fluorescence spectroscopy
SFE	solid-phase extraction
LC-ESI-MS	liquid chromatography electrospray ionisation mass spectrometry
SOES	School of Ocean and Earth Science
CPC	commercially produced cocktail
LPC	laboratory produced cocktail
EDTA	ethylene diamine tetraacetic acid, a strong chelating agent and anticoagulant
RQ	research question

Units	
Bq	Becquerel
kWh	kilowatt hour
ppm	parts per million
ppb	parts per billion
nm	nanometers
J/m^2	Joules per meter squared
mSv	milli Sieverts
mg/L	milligrams per litre
m/z	mass-to-charge ratio
Da	Daltons
M	Molar