HARMONISING METALWORKING FLUID FORMULATIONS WITH END-OF-LIFE BIOLOGICAL TREATMENT

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Department of Engineering Science

University of Oxford

BOONTIDA MEG UAPIPATANAKUL

Supervisor: Professor Ian P Thompson

Lady Margaret Hall

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Boontida Uapipatanakul, Lady Margaret Hall, University of Oxford.

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ABSTRACT

Metalworking fluids (MWFs) are coolants and lubricants, which are widely employed in metal cutting works. They are designed to be a long lasting product. Manufacturers have designed MWFs with lack of awareness of end-of-life disposal by including biocides, which make biological treatment challenging. Here, Syntilo 9913 was used as a case study to develop a cradle-to-grave product that was biologically stable in use but amenable to sustainable hybrid biological treatment at end-of-life. The product was reverse engineered employing factorial design approach based on *a priori* knowledge of the product components. From the combinatorial work, it was observed that chemical interactions can results in synergistic and antagonistic effects in terms of the toxicity and biodegradability. One of the major components of most MWFs are amines such as Triethanolamine (TEA). TEA does not biodeteriorate in single compound screening, but in combination with many other components TEA was found to cause "softening" of MWF formulations. Octylamine was found to be best for "bio-hardening" but it was not economically sustainable. Hence, the modified biocide-free synthetic MWF, Syntilo 1601, was reformulated with TEA, isononanoic acid, neodecnoic acid, Cobratec TT50S, and pluronic 17R40, which were resistant to biological treatment. Although, no change in the overall oxidation state of the MWF, metabolic activity did occur as breakdown products were observed. This suggested that both raw materials and metabolic breakdown products were recalcitrant. Thus, immobilisation agents were applied to aid further biodegradation by removing toxic bottleneck compounds. It was found that hybrid nano-iron and kaffir lime leaf performed similarly in removing chemical oxygen demand and ammonium from the system. Work in this Thesis demonstrated that the combined use of biological treatment and immobilisation agents effectively overcome the limitations of biological treatment alone by removing bottleneck compounds, which allowed greater COD reduction. This laboratory scale is a proof of principle, which needs to be tested at full scale.

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LIST OF ABBREVIATIONS

BAT: Best Available Technology

BOD: Biological Oxygen Demand

CFU: Colony Forming Unit

COD: Chemical Oxygen Demand

DDT: Dichlorodiphenyltrichloroethane

DfE: Design for Environment

DfS: Ecodesign for Sustainability

DNA: Deoxyribonucleic acid

E.coli: *Escherichia coli*

EU: European Union

FBR: Fluidised bed reactor

GAC: Granular activated carbon

GC: Gas Spectrometer

HP: Hypersensitivity pneumonitis

HPLC: High Pressure Liquid Chromatography

LB: Luria Bertani

LC: Liquid Chromatography

LCA: Life cycle assessment

Lum: lumincescence

MS: Mass Spectrometer

MTBE: Methyl t-butyl ether

MWF: Metalworking Fluid

NMs: nanomaterials

OD: Optical density

P.fluorescens: *Pseudomonas fluorescens*

P.putida: *Pseudomonas putida*

PBCs: Polychlorinated biphynelys

PBS: Phosphate Buffered Saline

PCA: Principle Component Analysis

PER: Post-Exposure Recovery

PHAs: Polyhydroxyalkanoates

PSS: Product Service Systems

PVC: Poly vinyl chloride

rlu: Relative light units

LIST OF ABBREVIATIONS

RO: Reverse osmosis

rpm: Revolutions per minute

SAR: Structure Activity Relationship

TCA: Tricarboxylic acid

TOC: Total organic carbon

UF: Ultrafiltration

UV: Ultraviolet

CHAPTER ONE

INTRODUCTION

CHAPTER ONE : INTRODUCTION

1.1 Introduction

The world population is continuing to increase at an unsustainable rate and is predicted to reach 9.38 billion people by 2050 (United States Census Bureau, 2013). In turn, natural resources are declining sharply, which means more energy is required to extract and process them from the environment (Lawrence, 2013). Consequently, there is growing concern with regards to global environmental issues such as climate change, pollution, and resource scarcity, resulting in increased legislation and responses from industry including a lifetime stewardship towards their products. Many industries are now moving towards sustainable "closed-loop" product designs (Chang *et al*., 2014; Sarioglu *et al*., 2014; Lawrence, 2013). A sustainable design approach helps to reduce raw material consumption, total waste production, and ensures effective resource recovery end-of-life (U.S. General Services Administration, 2014). For example, in 1998, Toyota was the first automobile manufacturer to establish a development and design division with ISO 14001. The objective for their engineers was to set achievable targets based on the concept of life cycle assessment (LCA) in order to enhance environmental performance. Consequently, Toyota's automobile designs allow the disassembly of many car parts in order to recycle or reuse at the end-of-life (Toyota Motor Corporation, 2013). In 2012, there were 34.5 million vehicles on the road in Great Britain alone, which 1.16 million were approaching their end-of-life. 88.1% of those were recovered to be recycled and reused (Date, 2014). Subsequently, on a global scale, 27 million cars could be recycled each year (U.S. Environmental Protection Agency, 2012). LCA is also one of the main concerns for

manufacturers. Long lasting products are the ultimate goal for both suppliers and consumers.

Currently, pollution is one of the world's biggest problems. For example, pollution caused a 50% fall in sperm counts in 50 years in many parts of the world such as India and Africa (Dindyal, 2003). Resources, especially fresh water, are in short supply throughout many areas of the world. In addition, a large number of pollutants lead to increased global warming, such as methane released into the atmosphere, which is 23 times more damaging than carbon dioxide (Themelis and Ulloa, 2007). Landfill and waste incineration produce greenhouse gases, for instance, methane, carbon dioxide, and toxic compounds, such as dioxins. Therefore, it is important to consider the final destination of the waste for effective disposal. Ideally, the aim is to recycle and reuse as much of the product end-of-life as possible. However, for the most part, this is almost impossible because the majority of current products have not been designed to enable a closed-loop economy. One of the advantages of the closed loop approach is that it reduces the release of potentially contaminating pollutants into the environment. There are many incidences where products have caused serious environmental problems, such as dichlorodiphenyltrichloroethane (DDT) made famous by Rachel Carson in 1962 (Carson, 1962). A further example was the explosion of a polyvinyl chloride (PVC) production plant, in 2004. The release of Dioxin and chlorinated compounds contaminated the environment. Additionally, these compounds are known to be human carcinogens (U.S. Chemical Safety Board, 2007). Such incidents have increased awareness of this hazard and motivated many industries to prioritise the concept of cradle-to-grave when designing products to aid self-disposal in the environment.

Bio-treatment is a cost effective and energy efficient treatment method. However, many products are designed to be biologically resistant, so there is a very low possibility of achieving this goal with current formulations. For this reason, it is necessary to solve the problem at the product design stage. Hence, this project was initiated in collaboration with BP-Castrol, who is a leading manufacturer of the metalworking fluid (MWF) products. The aim was to design an improved formulation, which had excellent durability in use, but was amenable to biodegradation at end-of-life. MWF was chosen as the model to develop the principals of sustainable design since it has been a topic of study within this research group of some years. They are also chemically complex but are formulated to an explicit specification, thus a good model to study the influence of chemical composition on a biological process. Current formulations are increasingly designed specifically to be toxic, recalcitrant, and unpalatable to microbial assimilation. This is ideal in terms of durability whilst in service since the product is intrinsically resistant to biodeterioration and loss of product function due to biological action. Conversely, because of their recalcitrance, they are not suitable for biological treatment at end-of-life.

MWFs are lubricants and coolants used in the metal working processes such as cutting, drilling, and constructing (BP Trading, 1969). The main functions of MWFs are to reduce the heat and friction produced from contact between tool and metal. In addition, they are also used for corrosion protection and rinsing equipment of metal chippings and sward (Gauthier, 2003). MWF generates significant health and environment concerns, because of their toxic nature (Chipasa, 2011). Currently, the operational life of MWFs are prolonged by the use of biocides and inclusion of recalcitrant chemical constituents. This makes them toxic and generally resistant to bio-treatment, and consequently disposal by sustainable means is difficult. Global consumption is approximately 2 million tonnes per year (Kline & Company, 2011). Current technologies for treating MWFs, such as ultrafiltration (UF),

vacuum evaporation, physical, and chemical treatment are capital and energy intensive, and generate sludge, which requires incineration or disposal to landfill, so they only provide a partial solution. Industries are, for this reason, developing products and manufacturing processes that are more sustainable. In particular, products which can be more effectively disposed of at the end-of-life are being designed, for example, vegetable oil based MWFs (Kuram *et al*., 2010; Shashidhara and Jayaram, 2010; Alves and Oliveira, 2008).

A pre-emptive approach was adopted for the work in this Thesis. A MWF was designed from first principles to be unsusceptible to premature biodeterioration by making it an unattractive nutrient source, whilst in contrast formulating it so that at end-of-life it is amenable to bio-treatment. In order to achieve that, research was based on a factorial design, which provides a powerful way of investigating the interactions between MWF components and identifying chemical combinations that are potentially useful in designing sustainable products that are both commercially competitive and biologically disposable. This is of great importance to MWF manufacturers because of the competitive advantage represented by these products. In addition, current disposal methods are not sustainable and therefore not in accordance with corporate responsibilities.

1.2 Aims and objectives

The aims of this study were to design from first principles a novel MWF formulation that was resistant to microbial attack during its operational life to prolong the product life, whilst being conductive to bio-treatment end-of-life sustainably by employing a chemical switch to remove key metabolic limitations. A further advantage is that biodegradation at

disposal potentially allows the MWFs waste components to be recovered potentially; for instance, enabling the organic material to be converted microbiologically to bioenergy. The long term goal motivating this research is that water (approximately 90% v/v) and materials can be recycled to help tackle the global scarcity problem. The final product is aimed to achieve cradle-to-grave principles when the MWF completes the full life cycle.

A factorial design approach was employed in this work to gain insight into the effects of the interaction between MWF components in terms of their "biohardening" or biodegradation. In depth understanding was obtained by screening the constituents of MWF components currently used by MWF manufacturers. MWF components were screened singly and in factorial combinations for toxicity and biodegradability. Through understanding the extent to which each of the chemicals breakdown during the biodegradation process, it was possible to determine the chemical pinch-points likely to hold up biotreatment. To overcome these pinch points, studies were undertaken to assess approaches for overcoming toxicity of pinch-point constituents by employing toxicity immobilisation agents.

1.3 Chapter summary

This thesis contains six chapters including, the introduction. The details of each chapter are summarised below:

Chapter 2: Literature review

This chapter describes the background information and review of current researches. This covers the overview information of MWF, regulation and legislation, current treatment methods, microbiology of MWF, sustainability, and eco-design of MWF.

Chapter 3: Reverse engineering of a commercial metalworking fluid product to formulate a more sustainable product

The studies detailed in this chapter focus on the reverse engineering of Syntilo 9913 with the view of determining which constituents of this MWF dictated vulnerability to biodeterioration and eventual amenability to end-of-life bio-treatment. Reverse engineer approach is the reverse engineering process, which identify the root of the problem. Syntilo 9913 was selected for this study since it contained only eight constituents; this made full factorial combinations of components manageable. In addition, this formulation's simplicity potentially made it easier to identify the key pinch-point constituents by liquid chromatography. Additional work was carried out to assess eighty generic individual components, which are commonly used to formulate MWF, for their toxicity and biological degradability to form a baseline understanding of each individual compound. Compounds were also tested in combination of increasing complexity to determine toxicity and biodegradation characteristics in particular interactions. Thereafter, the compounds found to make the formulation more susceptible to biodeterioration were removed and replaced by alternative constituents that had similar roles to produce a bio-hardened MWF and so fulfil the first requirement of this project for a durable in-use MWF.

Chapter 4: Disassembly of a MWF product to identify '"bottleneck"' compounds (Syntilo 1601)

In this chapter, the biodegradation process of reformulated synthetic MWF was examined. This was achieved by employing liquid chromatography to determine the fate of individual compounds and the accumulation of their breakdown products during biodegradation thus identifying "bottleneck" compounds. "Bottleneck" compounds are recalcitrant and have the potential ability to hinder or even stop biodegradation process feedback inhibition or toxic action. Therefore, there is a potential for employing these compounds in the future bio-resistant MWF formulation, without the need of biocides. Moreover, immobilisation agents and other technologies can be used to remove "bottleneck" compounds at the end-of-life to aid full biological treatment to achieve cradle-to-grave product design.

Chapter 5: Immobilising the toxic components in order to reduce microbial inhibition

This chapter applies the information gained from chapter 4 for further development. Hybrid technology was required to remove the "bottleneck" compounds, which were resistant to biodegradation, in order to aid complete biological treatment of MWF. This research investigated the optimum immobilisation agent to remove "bottleneck" compounds and/or adsorb toxicity of MWF or metabolic by-products.

Chapter 6: Conclusions and implications

This final chapter gives the overall conclusions of the research and discusses future work.

CHAPTER TWO

LITERATURE REVIEW

CHAPTER TWO : LITERATURE REVIEW

2.1 Metalworking fluids: The overview

Metalworking fluids (MWF) have been employed for the working of metals since ancient Egyptian times (BP Trading, 1969). They are a combination of lubricant and coolant, which is essential for the metal working processes and utilised in many industries; for example, cutting, drilling, constructing, and many other applications (BP Trading, 1969). Their main purpose is to reduce friction and remove heat produced in the metal working process (Cheng *et al*., 2005). In addition, they are also used for corrosion protection and rinsing of metal chippings and sward (Gauthier, 2003). With the employment of MWF on the surface of metal working operations, overall machine tool performance and productivity are improved. Metalworking fluids are used extensively in industries globally with a consumption of approximately two million tonnes per year (Kline & Company, 2011). In operation, MWFs are diluted with 90-95% water (CIMCOOL Fluid Technology, 2012), which consumes approximately 18 million tonnes of water worldwide per year. The largest user of MWF is the transport industry, including automotive (range from 76 – 2,839 m^3/day) and aerospace sectors. Despite the common uses of MWF, product development has been a gradual, empirically driven process. This has led to poor scientific understanding, particularly with regards to moving to more sustainable products, which needs to be addressed for product development to be optimised (Beercheck, 2012; Sutton and Mishra, 1994).

a).  (b). 

Figure 2.1: a). MWF employed in drilling process (Health and Safety Executive, 2015) b). in the grinding process (Northwest Aerospace Alliance, 2013).

2.1.1. Categories and functions of metalworking fluids

Metalworking fluids are classified into three groups, based on the oil and water content, namely straight oils, semi-synthetic, and synthetic (Cheng *et al*., 2005; Foltz, 2002; Wilbert, 1973).

Table 2.1: Classification of metalworking fluids (Derived from Byers, 2006).

Oil Content (%)	Type	
100 ↓ 0	Straight oils	Oil based
	Semi-Synthetic	Water based
	Synthetic	

- Straight oils: also known as ‘neat oil’. They are oil-based lubricants that contain no water.
- Semi-synthetic fluids: have greater water content and lower mineral oil content than oil based coolants. Typically, 30% or less in the formulation is mineral oil.

- Synthetic fluids: are the total opposite of the straight oils. They do not contain mineral oils but instead, synthetic organic compounds, often from plant sources and are used for lubricity.

Table 2.2: The advantages and disadvantages of each type of metalworking fluids

Type of metalworking fluids	**Advantages**	**Disadvantages**
Straight oils	• Good lubrication • Good coolant	• Poor corrosion control • May smoke • Short sump life
Semi-synthetic MWF	• Good lubrication • Good heat reduction • Good rust control • Long sump life • Clean	• Great tendency to foam in soft water • Unstable in hard water
Synthetic MWF	• Very clean • Excellent heat reduction • Excellent rust control • Long sump life • Convenient to use (transparent for worker to see the work) • Unaffected by hard water	• Poor physical lubrication • Difficult to treat • Can create foam in some applications

MWFs can also be classified by their application. There are four distinct uses: removal, formation, protection, and treatment (Glenn and van Antwerpen, 1998).

Table 2.3: The applications of each type of metalworking fluids

Types of Metalworking Fluids	Applications
Removal Fluids (cutting fluids/coolant)	Cutting, screw machining, tapping, boring, broaching, sawing, drilling, shaving, grinding, and milling
Forming Fluids	Operations involve changing shape or contour of metals (bending, stretching, pounding, and squeezing)
Protecting Fluids	Shield metal surfaces from air/water/other undesirable materials during storage and operation. Also, rust preventives (iron, steel, copper, aluminium, and brass)
Treating Fluids	Used in thermal processes, for example, quenching, where changes to meet application requirements are in the physical properties of the metal, e.g. hardness, elasticity

2.1.2. Components of metalworking fluids

The MWF components supplied by BP-Castrol for this study were used in a range of current and "in-development" Castrol products. MWFs usually contain between 8 to 30 components including biocides, corrosion inhibitors, emulsifiers, lubricating agents, anti-foam agents, metal passivation, and extreme pressure additives. (Canadian Centre for Occupational Health & Safety, 2005; Cheng *et al*., 2005; Byers, 1994; McCoy, 1994).

2.1.2.1. Biocide

A biocide is a substance that kills or inhibits living organisms (Health and Safety Executive, 2012). It can cause either bacteriostatic or biocidal effects. Antimicrobial agents work by inhibiting metabolism. An organism may recover from inhibition, but bactericidal effects are irreparable for the cell (Denyer and Hugo, 1991). Commonly used biocides are formaldehyde, triazine and oxazolidine. These are used in many products, such as foods,

insect repellents, hygiene products, and disinfectants. However, in the manufacturers' point of view, it is increasingly problematic to develop or use biocides since it is expensive to register new products and have them approved by governments. The cost is estimated to be $10 Million (Reisch, 2012). Furthermore, some biocides can no longer be used because of their toxicity or long term persistence (The Biocide Directive 98/8/EC, 16 February 1998).

2.1.2.2. Corrosion inhibitor

Corrosion inhibitors prevent the interaction between water/oxygen and a metal surface with acids and peroxides. They create physical barriers. Examples of corrosion inhibitor are calcium sulfonate, sodium sulfonates, fatty acid soaps, amines, and boric acid (Likhanova *et al.*, 2010, Anderson *et al*., 2003).

2.1.2.3. Emulsifier and surfactants

Emulsifiers are required in insoluble oil-water-based MWF to allow the emulsification between oil and water phases. Emulsifiers also reduce the tension on the fluids' surface. Examples of emulsifiers and surfactants are triethanolamine, sodium petroleum sulphonates, salts of fatty acids and non-ionic surfactants (Anderson *et al.,* 2003).

2.1.2.4. Extreme pressure agent

Extreme pressure agents are added to heavy duty machining MWF. They can tolerate the reaction of high temperature and pressure on the metal surfaces. Examples of extreme pressure agents are sulfurised fatty materials, chlorinated paraffins, and phosphorus derivatives (Anderson *et al.,* 2003).

2.1.3. Health impact of metalworking fluids

Metalworking fluid is a very toxic chemical mixture, which have both acute toxicity and chronic effects. Workers are exposed to the hazard by various ways such as inhalation of aerosols, skin contact while handling the operational process, and digestion. The most common pathway for the MWF hazard to travel to the workers is through the air. The contaminated air in the operational area may contain aerosols, thermal degradation products, tramp oil from the machine, and microbial toxins (Eisen *et al.*, 2001, Mattsby *et al*., 1989; Anderson, *et al*., 2009), which are particularly acutely toxic to respiratory system and may causes impairment of lung function, pulmonary diseases, asthma, hypersensitivity pneumonitis (HP), and chronically the workers can exposed to carcinogenic diseases, lung cancer (Khan *et al*., 2009; Eisen *et al.*, 2001; Mattsby *et al*., 1989; Iran *et al.*, 2005; Shashidhara and Jayaram, 2010; Byers, 2006). Bacteria and Fungi such as *Bacillus, Mycobacterium, Alternaria* and *Penicillium* are the possible causes for HP, which previous research found that *Mycobacteria* was discovered from the diagnosis. Moreover, there are thousands of chemicals products that produced from microbial community as toxins, which are found to be health threatening (Thompson and van der Gast, 2010). Another way for the workers to exposed to the MWF hazards is via splashes during handling tools, which may cause skin abnormalities such as dermatitis and irritation; and when digests MWF and may damage esophageal and pharyngeal membranes (Byers, 2006).

2.1.4. Regulation and legislation

With an increase, in environmental contamination during and since the industrial revolution, environmental laws have become necessary. For example, the Alkali Acts, was first introduced in 19th century (UK Environmental Law Association, n.d.). Moreover, the

increasing in water usage demand, Water Resource Act 1963 has been introduced to manage the water resource. Also, environmental legislation on water quality, EU Water Framework Directive (2000/60/EC), was established in order to control the quality standards of various types and usages of water and discharges. The Urban Wastewater Treatment EU Directive 91/271/EEC, regulates the collection and treatment of wastewater from homes and industries. In the UK, the Directive is implemented through the Urban Wastewater Treatment Regulations 1994 (Gray, 2010). Wastewater must meet minimum standards (see Table 2.4).

Water companies frequently use the Mogden Formula in order to determine the cost for discharging effluent into the sewers. It takes into account the Chemical Oxygen Demand (COD) and total suspended solid (TSS) values of the effluent in order to make a regional comparison of discharge streams. The Mogden Formula is then utilised according to need, with high scoring industrial effluent subjected to a greater rate of charge as a result of the higher COD and TSS values of the stream. MWFs are an example of high strength effluent which is subject to this higher rate (Bio-Wise, 2010). Note that the Mogden formula is heavily weighted towards COD, thus this measure is used as a key performance indicator throughout this work.

Equation 1: Mogden formula (The Water Services Regulation Authority, 2015; Walker, 2000).

$$\textbf{Charge per unit of effluent} = R + [(V + Bv) \text{ or } M] + B(Ot/Os) + S(St/Ss)^{7}$$

Where:

R = reception and conveyance charge [p/m^3]

V = primary treatment (volumetric) charge [p/m^3]

Bv = additional volume charge if there is biological treatment [p/m^3]

M = treatment and disposal charge where effluent goes to sea outfall [p/m^3]

B = biological oxidation of settled sewage charge [p/kg]

Ot = Chemical oxygen demand (COD) of effluent after one hour quiescent settlement at pH 7

Os = Chemical oxygen demand (COD) of crude sewage one hour quiescent settlement

S = treatment and disposal of primary sewage sludge charge [p/kg]

St = total suspended solids of effluent at ph 7 [mg/litre]

Ss = total suspended solids of crude sewage [mg/litre]

Table 2.4: Mandatory minimum wastewater standards (Blöch, 2005)

Parameters	Value (Concentration)	Value (% reduction)
Biological Oxygen Demand*	25 mg/L	70 - 90%
Chemical Oxygen Demand*	125mg/L	75%
Nitrogen ** Plants of 10000-100000 p.e.	15mg/L	70 – 80%
Phosphorus ** Plants of 10000-100000 p.e.	2mg/L	80%

* 24 hour average; either concentration or percentage of reduction shall apply

** Annual averages, either concentration or percentage of reduction shall apply

However, most industrial wastes are heavily polluted and therefore, some are required to be pre-treated on-site. Waste can be disposed off-site to sewer as trade effluent or via a licensed waste contractor. However, this is not cost effective and does not utilise best available technology (BAT) principles and so other disposal routes are being utilised. Some manufacturers pre-treated their waste on-site employing methods such as chemical separation, evaporation, ultrafiltration, hybrid systems, and biological treatment (Bio-Wise, 2010).

EU legislation - EU Directives allow for onsite treatment of waste thus opening up opportunities for water recycling and resource recovery from waste MWFs (Microbial Solutions Ltd., 2012). Beside the waste, there is much concern on the production aspect as it is the root cause of the pollution. The producers often lack of awareness and are concerned about producing long lasting product have priority over cradle-to-grave design, therefore, there has been increased in the legislation on the product and the chemical itself such as The Biocide Directive 98/8/EC on biocidal products.

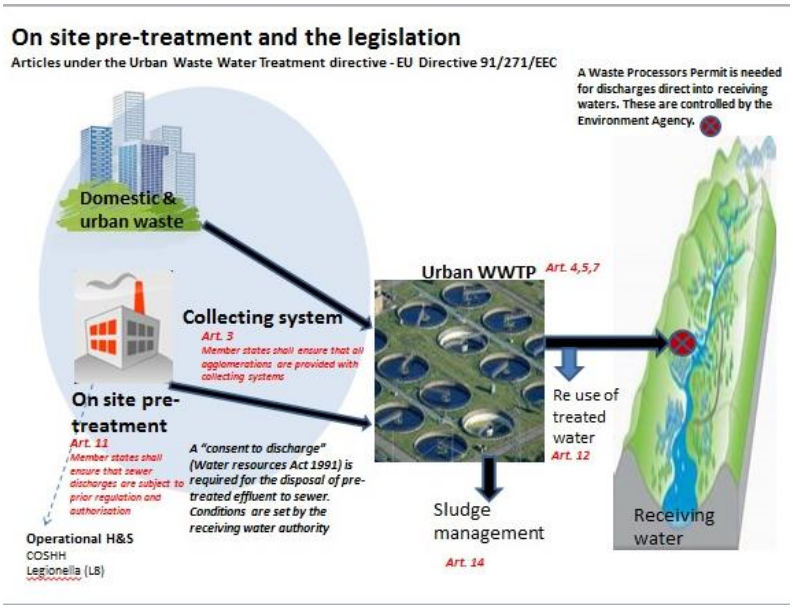

Figure 2.2: On site pre-treatment framework (EU-Directive 91/271/EEC)

(Microbial Solutions Ltd., 2012).

2.2 Treatment of waste metalworking fluids

Currently, there are several methods to treat operationally exhausted metalworking fluids; ultrafiltration (UF), vacuum evaporation, physical and chemical treatment. At the beginning of the 21st century, the main treatment methods for MWF were chemical and physical treatments, and the spending on the treatments was approximately £16 million per year (Cheng *et al.*, 2005; Ji *et al.*, 2004; Portela *et al*., 2001; Viraraghavan and Methavan, 1990; Kim *et al*., 1989; Sutton *et al*., 1989). These methods are capital and energy intensive, only partially effective, and are not sustainable. The main reason why biological treatment of MWF has attracted increasing attention is because it is sustainable and also scalable. Hence, in recent years, interest has grown in the biotreatment of waste fluids by applying the natural process of microbial degradation (Dash *et al.*, 2009). More interestingly, it is commonly to combine with processes such as activated sludge and/or ultrafiltration membrane technology (Cheng *et al*., 2005).

2.2.1. Chemical treatment

Chemical agents, such as lime, sodium, alum, aluminate, and charged polymers are added to the waste for splitting the oil/water emulsions. In spite of the recovered water being recyclable, the process residues require further treatment, such as incineration, which is costly (Chipasa, 2011; Tchnobanoglous *et al*., 2004). Thus, chemical splitting simply concentrates and exacerbates the problem. In addition, this method is not suitable for synthetic fluids because as there is no emulsion to split in these products. The examples of chemical treatments are the use of inorganic chemicals, use of cationic/anionic organic compounds, chemical oxidation, and hydrothermal oxidation (Cheng *et al*., 2005). Chemical Oxidation, as an example, is described in detailed below.

2.2.1.1. Chemical oxidation

After undergoing secondary treatment, several oxidizing agents may be combined or used individually in order to reduce the BOD and/or COD values of the waste stream. These may also be used to reduce organic or nitrogen-containing compounds within the stream. Examples of oxidants frequently used in this process include sodium hypochlorite, ozone, hydrogen peroxide, either alone or in combination with either ferrous sulfate, which induces Fenton's reaction, or UV radiation. UV radiation may also be combined with ozone to further degrade contaminants (Byers, 2006).

2.2.2. Physical treatment

Physical treatment causes a change in the physical properties and separates liquid and solid phases by applying heat or employing membranes. However, this method is capital and

energy intensive. Membranes also have a short life-span, and are costly to replace. Examples of physical treatments are evaporation, membrane separation, microfiltration, ultrafiltration, reverse osmosis (RO), and peat adsorption (Cheng *et al*., 2005). Two of the examples are described in detailed below.

2.2.2.1. Ultrafiltration

Ultrafiltration is a process whereby MWF is pumped through a semi-permeable membrane, which only allows water to filter through, leaving the oil and surfactants behind. This method is easy to use and effective for continuous and batch processing. However, the drawback is that the life of the membrane is approximately 1.5 to 3 years, the capital cost is high, it generates a high carbon footprint, and there are limitations of fluid types it can treat effectively. For instance, it is unsuitable for synthetic waste fluids because soluble compounds pass through the filter and so are still present in the permeate (Chipasa, 2011; Tchnobanoglous *et al*., 2004).

2.2.2.2. Evaporation

Thermal energy is applied to evaporate water and concentrate the waste. However, this method is not always efficient. Although, this method is simple but the drawbacks of this method in addition to cost are that air pollution and foam can be created (Byers, 2006).

2.2.3. Biological treatment

An alternative to current technologies is biological treatment. Biological treatment of waste is a “Green” technology, avoids or limits the use of hazardous chemical additives (U.S.

Environmental Protection Agency, 2012), is energy efficient, environmentally friendly, and allows for water recycling. However, modern MWF formulations are designed to be microbiologically resistant and so typically do not fully degrade during bio-processing. There are two broad mechanisms for inhibiting microbial growth and thus extending the life of the MWF product, toxicity, and biodegradability/bioavailability. High recalcitrance and low biodegradability are desirable characteristics in an operational metalworking fluid. In contrast, low toxicity and high biodegradability are desirable characteristics for end-of-life product disposal and recycling. Despite the slower treatment rate, the treated water and sludge are recyclable, which is a significant advantage. Microbial degradation in aerobic conditions can remove chemical oxygen demand more rapidly and comprehensively than anaerobic treatment and so was the focus of this project (Thompson and van der Gast, 2010; Connolly *et al.*, 2006). In addition, overall performance can be enhanced by employing synthetic biology to specify certain species (van der Gast *et al*., 2004). The examples of biological treatments are Aerobic activated sludge (AAS), Aerobic sand fluidised bed reactor (FBR), Anaerobic activate sludge, Anaerobic granular activated carbon (GAC) FBR, and Reed wetland (Cheng *et al*., 2005).

2.3 Microbiology of metalworking fluids

Metalworking fluids are largely oil emulsions and as such attractive nutrient sources for bacteria, and fungi. Air borne microorganisms enter the machine lathe and colonise the internal surfaces, forming biofilms. Moreover, MWFs in operation are made up of 90-95% of water (see section 2.1), MWFs are contaminated by microorganisms from the source of water and from atmospheric deposition. Microbial action causes deterioration of the MWF (e.g. pH changes), which affects the fluid's performance and necessitates premature disposal. Therefore, antimicrobial agents are introduced into the MWF to prevent bacterial

proliferation (Cheng *et al.*, 2005; Rossmore and Rossmore, 1991; Rossmore, 1981). This has been of much interest since the mid-20th century (Cheng *et al*., 2005; Baker *et al.*, 1983). MWFs contain viruses, yeasts, fungi, and protozoa in addition to bacteria. This work focused on bacteria because they are the most common contaminating taxa present in MWFs.

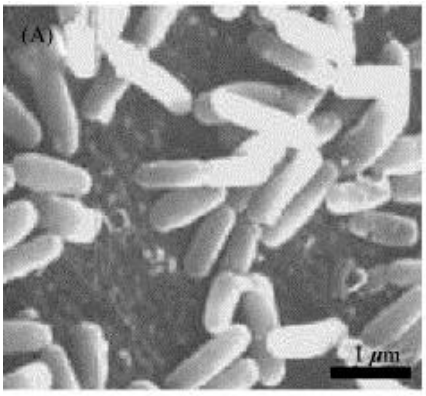

Figure 2.3: Scanning electron microscopy of normal *E. coli* cells. (Cho *et al*., 2005)

2.3.1. Biodeterioration and biodegradability

Biodeterioration is the gradual deterioration of products by biological activity, which lowers the quality of MWF performance. For example, viscosity may change and fluid pH is lowered due to the fermentation of acidic products, which causes corrosion and leaks in the system (Bakalova *et al*., 2007; Rao and Srikant, 2006). Wastes are nutrient rich for bacteria, macro nutrients such as Carbon, Nitrogen, Sulfur, and organic compounds are abundant (Alves and Oliveira, 2008). Bacteria, for example, *Escherichia coli (E.coli)* use a carbon and nitrogen source to grow. As a product, Carbon dioxide (CO_2) and water (H_2O) are produced from the biodegradation process as shown in the reaction below. The purpose of the biocide is to prevent these natural processes.

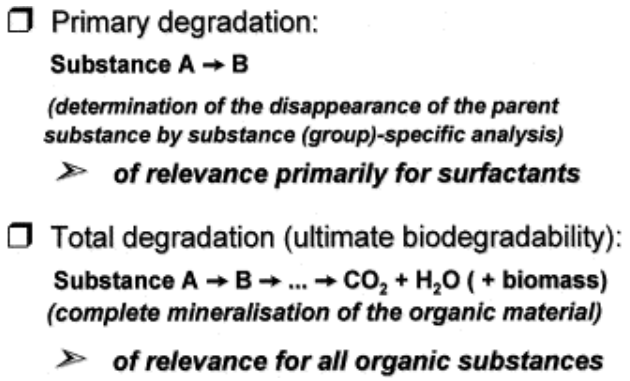

Figure 2.4: Reactions of degradation process (Willing, 2001).

Hence, bacteria play an important role in completing the carbon cycle as represented in Figure 2.5, including biotransformation and recycling key nutrient, and water.

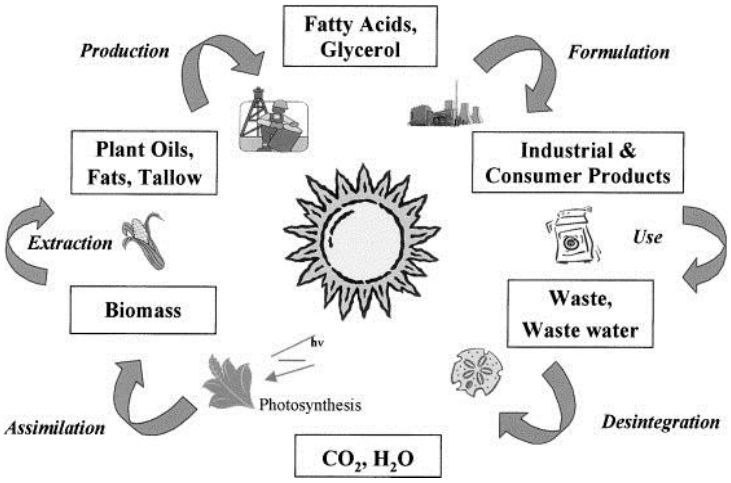

Figure 2.5: Carbon cycle (Willing, 2001).

2.3.2. Aerobic and anaerobic conditions

Microorganisms required a carbon source as a main food for growth (Tchnobanoglous *et al*., 2004). The metabolic pathway for metabolism is greatly affected by the presence or absence of oxygen. In both case, the products of degradation are carbon dioxide (CO_2),

water (H_2O), nitrates, sulphates, and biomass (Tchnobanoglous *et al*., 2004). Comparison of aerobic and anaerobic condition is made in Table 2.5.

Table 2.5: Comparison of aerobic and anaerobic degradation (derived from Bio-Wise, 2010).

Aerobic condition	**Anaerobic condition**
Waste effluent is degraded at a greater rate under aerobic conditions	High COD/BOD components are degraded more quickly meaning that waste-streams are more easily digested
Complementary anaerobic systems break down difficult components into compounds, which aerobic bacteria can then digest more easily and rapidly	Different and more complicated components can be broken down more easily
High digestion rate	Low rate of digestion
Low upfront costs	High upfront costs to control conditions
Aerobic processes required aeration	External heat sources may be required if environmental temperature is low
More robust and less prone to upsets	More prone to toxic shock, which can lead to total or partial system failure
A simpler, proven technology	The underlying technology requires more involvement from skilled personnel to operate properly
By-products are benign being free of methane, unpleasant odours or corrosive substances	The production of methane as a by-product is problematic as it has to be scrubbed or burnt
Greater amount of biomass production, which has to be dried out	Drying is easy due to the less amount of biomass production

2.3.3. Metabolic pathway

Metabolism is a process of chemical transformation into smaller or larger molecule, which called the process of catabolism and anabolism, respectively. These processes occur within

the cells of living organisms. Most organisms are made up of three basic classes of molecules: amino acids, carbohydrates, and lipids. They can either construct into more complex structure such as DNA or they can breakdown to smaller compound and create the source of energy.

2.3.3.1. Krebs cycle

Krebs cycle is also known as tricarboxylic acid (TCA) cycle. This is a common pathway of metabolism. Protein, carbohydrate, and fats are all eventually broken down and enter TCA cycle as shown in Figure 2.6. This process occurs in mitochondria of the cell, which consumes oxygen and yields carbon dioxide, hydrogen ion, and energy (see Figure 2.7).

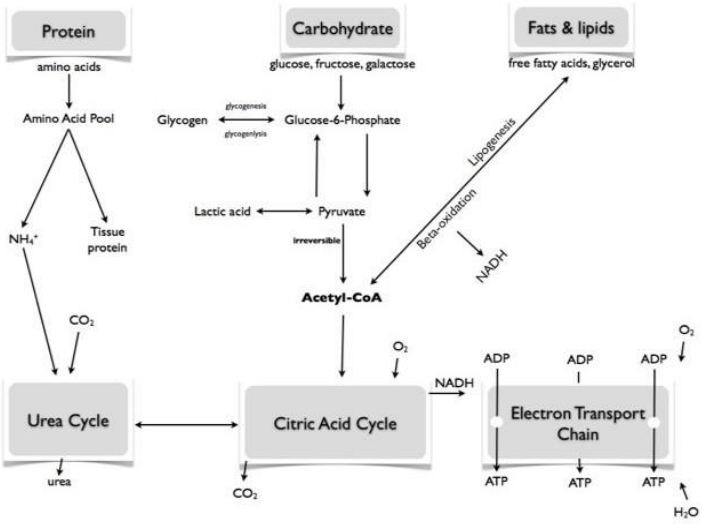

Figure 2.6: Process of metabolism

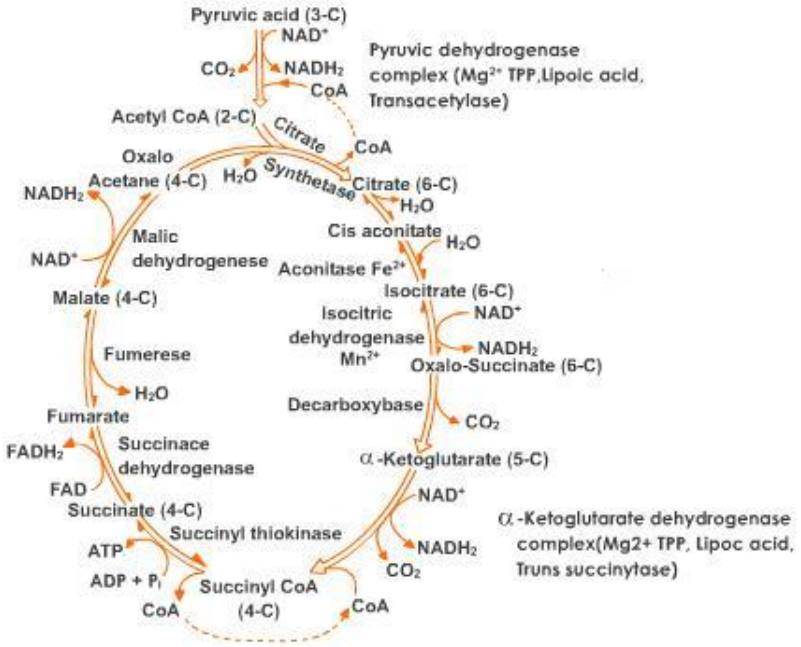

Figure 2.7: TCA cycle

2.4 Sustainability and eco-design

Harmonising the formulation of any chemical product to optimise performance, disposal and recycling is a significant challenge for biological treatment development. Manufacturers design most products without considering disposal at their end-of-life, which is a barrier to achieving a closed-loop economy. Much waste requires high carbon footprint treatment methods, such as chemical and physical treatment because biological treatment is not possible. However, biological treatment is preferred as it requires lower energy consumption than current technologies. Moreover, at the end of treatment, materials are often reusable and recyclable (Figure 2.8). Therefore, it is of interest to many researchers and industries to design sustainable products. For example, in North West Europe, synthetic base stock MWF formulations, such as vegetable oils are viewed favourably (Glenn and van Antwerpen, 1998). Synthetic formulations are more predictable in terms of treatment and also crude oil is fast becoming scarce as a natural resource. However, synthetic MWF is not favourable for the biodegradation, therefore,

immobilisation agent stage is introduced to the process to aids biological treatment as shown in Figure 2.9.

Figure 2.8: Biological treatment promotes close-loop product life cycle

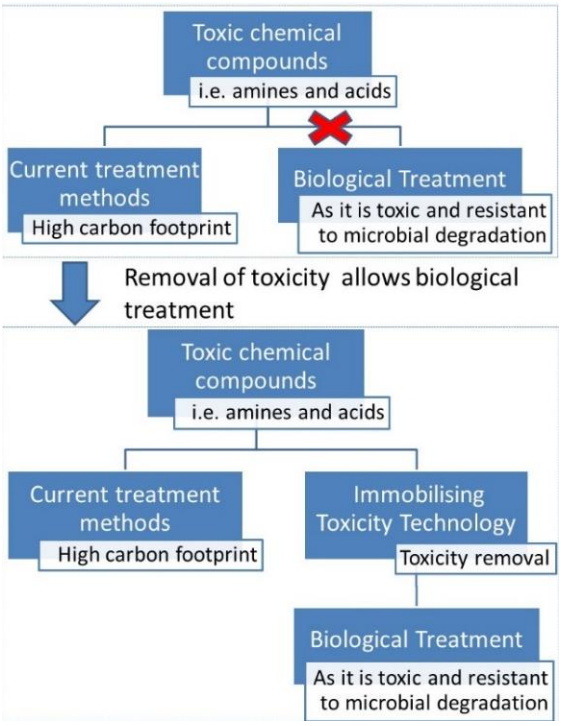

Figure 2.9: How hybrid technology can aid biological treatment, which leads to cradle-to-grave design

There are two main approaches being studied, traditional and radical approaches. Traditional approaches include design such a Design for Environment (DfE) and

Ecodesign for Sustainability (DfS) in order to optimise existing products, processes, and businesses (Brezet and Hemel, 1997; Gertsaksis *et al.*, 1997; Charter and Tischner, 2001; Lewis and Gertsakis, 2001; Crul and Diehl, 2006). Radical approaches such as Product Service Systems (PSS) and System Innovation for Sustainability focus on developing new products and services with higher environmental gains (Brezet *et al*., 2001; Charter and Tischner, 2001; Manzini and Vezzoli, 2002; Mont, 2004; Tukker and Tischner, 2006a, Tukker and Tischner, 2006b).

Factorial Design has been extensively used in various researches e.g. the pharmaceutical industry. It is an experimental design for multiple independent variables in a single study. This study has applied factorial design to reverse engineer one of the most common synthetic MWF, Syntilo 9913, to gain an insight of chemical interaction toward bio-hardening MWFs to prolong product life in the machine.

2.5 Summary

MWFs are a combination of lubricant and coolant employed in metal working processes mainly to reduce friction and heat. MWFs have four types and the generally composed with lubricating agents, corrosion inhibitors, emulsifier, and biocides. They are known to be toxic to both organisms and environment. Hence, the regulations have been tightened and most industrial effluents are required to be pre-treated before it can be disposed to the sewer, or the environment.

MWFs are designed to be biologically resistant to prolong the product life in operation, which leads to difficulty in biological treatment at the end-of-life. The current treatment methods such as physical and chemical treatments are cost and energy intensive. Sustainable designs have, therefore, become attractive to make the product greener, in this case, synthetic MWF, Syntilo 9913, was employed as a case study. Factorial design and reverse engineering approaches were applied to reformulate Syntilo 9913. Then, the metabolic degradation pathway of reformulated MWF, Syntilo 1601, was studied in order to gain an insight into the "bottleneck" compounds that stop the biodegradation process. Hybrid technology was then applied to remove toxicity/"bottleneck" compounds and aid further degradation to meet legislation. The main focus of this study was to design MWF that has greener formula with hybrid solution, which enable biological treatment at the end-of-life. This would allow water and materials to be recovered and recycle.

CHAPTER THREE

REVERSE ENGINEERING OF A COMMERCIAL METALWORKING FLUID PRODUCT TO FORMULATE A MORE SUSTAINABLE PRODUCT

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3.1 Introduction

This chapter includes three-steps-experiment, which are single screening, combinatorial studies, and amine substitution. In each experiment, there are three tests involved; toxicity, post-exposure recovery, and degradation tests. In toxicity and post-exposure recovery tests, *E.coli* HB101 biosensor was employed, whereas mixed bacteria consortia were employed in degradation test. Results are converted into percentage in order to compare across the experiments.

3.1.1 Single screening

In order to design new formulations which enable more sustainable management it was essential to start by accessing the characteristics of the current product range available to manufactures of MWF. Working with our commercial collaborators, BP-Castrol, 80 generic metalworking fluid (MWF) constituents were studied. They are generic chemical compounds used in formulating MWF for the current Castrol range. These consisted of 21 amines (corrosion inhibitors), 13 acids (coating agents/lubricity additive), 35 oils (lubricity additive/surfactants), 1 biocide (antimicrobial agent), and 5 polymers (lubricity additive). It is important to note that one compound can have multiple functions. For example, compound A12, Triethanolamine, has 3 main roles; a surfactant, emulsifier, and corrosion

inhibitor. The aim of the detailed analysis was to characterise and categorise 80 chemical constituents, each of which was employed in a range of combinations to formulate MWF in terms of their toxicity towards bacterial cells, retarding/stimulating their growth, and general recalcitrance. To achieve this objective, each compound was tested individually and in combination with other constituents, specifically to the biosensor *E.coli* HB101 and a bacterial community adapted to MWF exposure and employed as a surrogate of general community response to chemical exposure. This was done by testing several responses of the microbial community, once exposed to chemical components including: growth inhibition, toxicity towards cells including biosensors, and post-exposure recovery. The overall goal was to exploit this baseline information to develop new MWF formulations with predictable in use and end-of-life bio-treatment performance. This places the corresponding industry in an improved position in terms of the durability and sustainability of their products, so proving more cradle-to-cradle information with regards their products. For instance, through this work, it was also found that a MWF could be formulated without the uses of biocides but that were still be resistant to microbial colonisation.

A combination of techniques was used to assess the toxicity and biological degradability of a panel of eighty individual MWF components representing the full palate of options available to formulation chemists. However, most MWF typically only contain between 8 and 20 of these constituents. As mentioned, the compounds were tested singly and in combination (see section 3.1.2); initially with just two combinations but then with increasing complexity up to 8 chemicals. Growth assays were used to determine whether, and to what extent, each compound could be utilised as a sole carbon source by MWF acclimatised bacteria. Much research has monitored biodegradation process by providing sample as the sole carbon source (Coleman, *et al*., 2002; Nakajima-Kambe *et al*., 1995). Nakajima-Kambe *et al.* (1995) has demonstrated that polyurethane degraded to adipic acid

and diethylene glycol by measuring bacterial growth on sole carbon source. Toxicity was assayed using a bioluminescent bacterial sensor, which is an analytical detector of biological responses expressed as luminescence, in a two-stage test involving both exposure: toxicity test (Figure 3. 10) and post-exposure data to distinguish between inhibitory and lethal effects (Table 3.9 shows raw data for post-exposure recovery).

3.1.2 Combinatorial studies

Combinatorial studies were carried out to identify component interactions that produce "hardening" or "softening" effects, which could facilitate biohardening or biotreatment, respectively. To be specific, a "hardening agent" or constituent in this respect is one which when present on its own or in combination with another chemical constituent inhibits or slows down biodegradation activity, and thus contributes to extending the life of the MWFs. In contrast, a "softening agent" is defined here as one whose presence contributes to making the total MWFs more susceptible to premature biodegradation/biodeterioration.

The compounds selected for this part of the study were the individual components used in the Syntilo 9913 formula. This formula was selected because Syntilo 9913 is a synthetic MWF, which contains only eight chemical compounds. This allowed the full factorial, ignoring order, for the combinatorial approach to be manageable within the timeframe of this work.

The eight individual components represent the range of functional components, which together perform as an MWF. For example, A12 (Triethanolamine, TEA) was selected

from 21 possible amines options. Combinations of individual MWF constituents were tested by employing a factorial design of increasing complexity initially with two compounds progressing to eight compounds in a 96-well microtitre plate format. The formulation of the Syntilo 9913 product is as shown in the Table 3.2. BP-Castrol Syntilo 9913 flagship is a synthetic coolant, which contains a mixture of surfactants, corrosion inhibitors, lubricants, coating agents, and an antimicrobial agent. It is commonly used in the aerospace and automotive industries. This product offers excellent lubrication and cooling properties. This was developed specifically by our commercial partners for 'heavy duty machining of aluminium and other aerospace alloys'. (Castrol Limited, 2008).

Table 3.1: Typical physical and chemical characteristics of Syntilo 9913 metalworking fluid (Castrol Limited, 2008).

Concentrated Metalworking Fluids	
Appearance	Transparent, Yellow
Density	1.056 - 1.078 kg/m^3
Mineral oil content	0 Vol %

Metalworking Fluids in Solution	
Appearance	Transparent
pH (5%)	7.2 – 7.6
Factor refractometer	1.4

Table 3.2: Formulation of Syntilo 9913 metalworking fluid (BP-Castrol Limited, 2012) (see Appendix I for compound code).

Compound code	Class of chemical	Application	Concentration (%)
A12	Amine	Surfactant/Emulsifier/Corrosion inhibitor	15.0
B3	Acid	Corrosion inhibitor	1.0
B9	Acid	Lubricant	2.5
B10	Acid	Coating agent/Corrosion inhibitor	5.0
B30	50% Tolyltriazole	Corrosion inhibitor	1.0
B31	Polymer	Lubricant	15.0
B32	Polymer	Lubricant/coating agent	15.0
D1	Biocide	Antibacterial agent	0.5

3.1.3 Amine substitution

This experiment was carried out as an extension of the combinatorial studies (see section 3.1.2). Preliminary work identified interactions, which resulted in unexpected interactions between MWF constituents. For example, the presence of two easily degraded components when in combination, actually found to inhibited the overall rate of biodegradation. In Figure 3.16, those instances are highlighted, where two individual compounds determined from the single component screening were found to be non-toxic, and easily biodegraded. Such studies could identify instances where the individual constituents stimulated bio-deterioration, so shortening the expected MWFs' life expectancy. In these instances, such components would be replaced with more recalcitrant components. In contrast, there may be instances where single compound analyses predict that two compounds should degrade readily but when combined, may in fact prove to be resistant to biodegradation.

Biocide-free MWFs (based on Syntilo 9913 product) were also tested. In this case, the rationale was that by making a MWF unattractive as a nutrient source to colonising microbes, there would be no necessity for the addition of a biocide. It would be beneficial to industries if biocide-free MWF formulations could be designed, since biocides are expensive to test and register as new products with national authorities (i.e. a typical cost of $10 million and 5 years of paperwork) (Reisch, 2012). Furthermore, some biocides can no longer be used because of their toxicity or long-term persistence in the environment, which restrict their use under the biocide directive (The Biocide Directive 98/8/EC, 16 February 1998).

3.1.4 Toxicity measurement

Ecotoxicity is defined as "the branch of toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial, in an integral context" (Truhaut, 1977). Therefore, it is important to comprehend the critical concentration of chemicals which affect the environment and ecosystems; as if one organism is affected, other organisms in the web may be impacted (Koeman, 1998; Bardgett, 2005; Procter and Gamble, 2005; Cole *et al.*, 2006). Well-established bioassay methods were used for this work. The bacterial bioassay approach is the most effective method because it is time efficient, easy to conduct, cheap, and applicable to a wide range of pollutants, which were the main rationales for its usage here (Koskella and Stotzky, 1997; Tiensing *et al.*, 2002).

Bacteria respond rapidly to changes in environmental conditions in their immediate environment. Whole cell bacterial biosensors, such as *E.coli* HB101 strain and *E.coli* dH5α

strain can be used in the 'canary in a cage' approach by acting as a surrogate for higher organisms in toxicity testing (Madigan *et al.*, 2000; White *et al.*, 1998; Kelly and Harwell, 1989). Biosensors report the integrated effects of mixed chemicals, and are useful in measuring the toxicity of soluble substrates at low concentrations. To perform as a biosensor the *E.coli* has been transformed with a plasmid containing bacterial bioluminescence (*lux*) genes taken from *Vibrio fischeri.* The *lux* operon has been modified by removal of the regulatory I and R sequences so that CDABE genes are expressed constitutively. This makes the transformed cells constantly produce light. The light reaction is directly related to intracellular adenosine triphosphate levels (ATP), the cells energy currency. Lower energy levels are thus reflected by decreased light output (see Figure 3.1). The light output is measured by a photomultiplier in a standard plate reader.

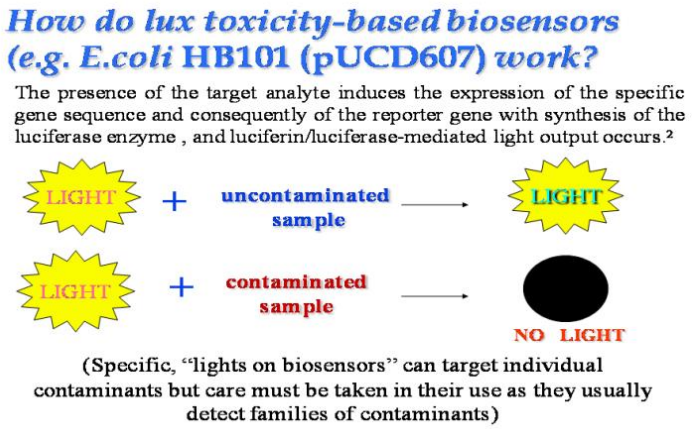


Figure 3.1: Illustration of how *lux*-based biosensors work (Mwinyihija, 2011).

3.1.5 Aims and objectives

The overall aim of this study was to formulate a new MWF, which had a more predictable performance in terms of resistance to biodeterioration and potential end-of-life bio-treatment. This was achieved by screening the individual chemical constituents of current MWFs, and of those in development. Synthetic MWF, Syntilo 9913, formulation were

screened both singly and in combination, in terms of toxicity and degradability. Single compound responses were used to predict the fate of compounds when in combination. Combinations that were found to be more or less toxic or biodegradable than predicted by the single compound model were studied in further detail. Such detailed analysis identified the pinch-point chemical components, which determine susceptibility biodegradation and resistance to bio-deterioration. Information can be exploited to design MWF that are more compatible with sustainable practices in terms of reducing the need for reliance on biocides and end-of-life biotreatment.

3.2 Materials and methods

3.2.1 Plasmid DNA purification and transformation

The plasmid vector pUCD607 contains the *lux* genes, which produce luminescence and therefore can act as a biosensor. Briefly, luminescence is related to cell energy levels. Any toxic insult that reduces energy levels is reflected in a reduction in luminescence. Luminescence is usually measured in a spectrophotometer. In fact, this biosensor produces light in the visible range and at levels detectable by the naked eye. In order to have a comparable candidate, *E.coli* dH5α was chosen. *E.coli* dH5α is a competent cell for DNA transformation, with the transfer of the plasmid from *E.coli* HB101, enabling it to act as a biosensor. It is reported that *E.coli* biosensor has high sensitivity to a range of organo-xenobiotics (Strachan *et al*., 2001; Sinclair *et al*., 1999). This experiment provides an opportunity to increase biosensor options in the future. The plasmid in *E.coli* HB101, pUCD607, was extracted by using a miniprep method. Plasmid DNA purification was undertaken by using the QIA Prep Spin Mini-prep Kit and a Micro-centrifuge. The

protocol was followed according to the handbook; plasmid purification, cell transformation, then recovery on a plate and tests for light output (QIAGEN, 2006).

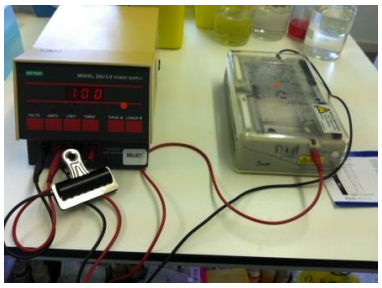


Figure 3.2: Gel electrophoresis at 100 Volts

Transformation of *E.coli* dH5α was confirmed by gel electrophoresis. The results from gel electrophoresis showed that *E.coli* dH5α has the same plasmid as *E.coli* HB101, thus allowing the former strain to be used as a biosensor. The product was stored in the glycerol stock (15% v/v) in screw cap cryotubes at -80ºC (U410 premium, New Brunswick Scientific).

3.2.2 Selection of test microbial strains and agar plate for toxicity testing

Luria Bertani (LB) and Nutrient agars are standard media commonly used to grow bacteria in the laboratory. In this experiment, two types of agar were compared, and the optimal agar was selected for use in all experiments. Both types of agar; LB, Miller agar (40g/L, Fisher Scientific) and Nutrient agar (28g/L, OXOID) were dissolved in distilled water (Direct-Q UV3, Millipore) with the addition of D-glucose anhydrous (5g/L, Fisher Scientific) and then autoclaved (Classic, Prestige Medical). Ampicillin stock (25µg/L) was prepared by dissolving 0.5g of ampicillin (Sigma) in 10mL deionised water, which was then sterilised using a 0.2µm sterile filter (Thermo Scientific) and syringe (BD Plastipak). Subsequently, 250µL of the aqueous solution was dispensed into 1.5mL centrifuge tubes

(Eppendorf AG) and stored at -80ºC (U410 premium, New Brunswick Scientific). Sterile ampicillin stock was added after the liquid agar was autoclaved and the temperature of the liquid had reduced to approximately 40ºC. Once it was well mixed, 25mL of the agar was poured into the Petri dishes (Fisher Scientific). The agar plates were dried in a laminar air flow cabinet (Bio2^{+}, Envair Ltd.) to remove the condensed water. This prevents swarming growth by motile organisms and bacterial lawn formation.

Two bacterial strains, *E.coli* HB101 and *E.coli* dH5α were compared in order to select the optimal biosensor strain for the experiment in terms of light production. *E.coli* was chosen because it is straightforward to culture in the laboratory. Both strains are highly competent cells in terms of their ability of to take up extracellular DNA from their environment, but they have a wide variation in the amount of extracellular polysaccharide produced. The amount of extracellular polysaccharide present has a potential effect on the toxic response. *E.coli* HB101 and *E.coli* dH5α were streaked out on the solidified LB and Nutrient agar plates once cooled and dried, using a sterile spreader (Sterile L shaped spreader, Microspec). The cultures were grown in a static incubator (Genlab) at 30ºC overnight. The plates were kept at 2ºC throughout the experimental period. This procedure was repeated once a week for the remainder of the research period to maintain the plasmid in the presence of ampicillin for both *E.coli* HB101 and *E.coli* dH5α strains. In the absence of the antibiotic, the plasmid would not be maintained by the bacterial cell, thus those bacteria strains would not be able to luminesce and be used as a biosensor. Furthermore, the luminescence was monitored in the dark room once a week for 12 weeks. As shown in Table 3.3 below, it was found that *E.coli* HB101 strain produced more light than *E.coli* dH5α in both LB and Nutrient agars. The greater signal makes detection easier; hence *E.coli* HB101 was selected as the biosensor for this research and LB agar was selected to use to grow the culture for the rest of the experiment.

Table 3.3: Comparison of the light production of *E.coli* HB101 and *E.coli* dH5α in LB and Nutrient agars

Bacteria strain	**LB Agar**	**Nutrient Agar**
E.coli HB101	100%	80%
E.coli dH5α	70%	70%

3.2.3 Determination of mid-exponential phase

Distinguishing tests; toxicity and Post-Exposure Recovery (PER) tests are vital as they differentiate lethal from inhibitory effects of chemical exposure to bacterial cells. PER test was used to identify the level of toxicity when the biosensor does not show any signal and appeared to be dead, but subsequently can be regrown on the agar plate. The toxicity and PER experiments utilising *E.coli* were most effectively undertaken when bacteria were at the mid-exponential phase, because the membrane is at the most sensitive phase of growth; therefore, it is the optimal phase to observe the effects that compounds have on the cells. To identify the mid-exponential phase, typical growth and luminescence growth curves were produced. Growth and luminescence curves were obtained by incubating the *E.coli* biosensor in the growth media and plotting optical density readings against time. The experiments were carried out in triplicate.

LB Broth (growth media) was prepared by autoclaving a mixture of 10g/L Tryptone (OXOID), 5g/L yeast extract (Fisher Scientific), 5g/L sodium chloride (Fisher Scientific), 1g/L glucose in deionised water. Ampicillin stock (25µg/L) was added after the solution had cooled down to prevent thermal degradation of the antibiotic. *E.coli* HB101 was inoculated into 5mL sterile LB Broth and kept overnight in a shaking incubator at 35ºC and

120rpm (Innova 44, New Brunswick Scientific). After 16 hours, the inoculum was transferred into 100mL sterile LB Broth. Optical density (OD) and luminescence were recorded every 30 minutes using a UV spectrophotometer (UV-1800 Shimadzu) and a micro-titre plate spectrophotometer (Synergy HT, BioTek). All processes were performed under aseptic conditions.

Figure 3.3: Micro-titre plate spectrophotometer

From Figure 3.4 and Figure 3.5 below, it can be seen that the optimal time to conduct the experiment was 8 hours after inoculation., when *E.coli* HB101 was at the mid-exponential phase and was producing light because the cell membrane is at the most sensitive phase of growth; therefore, it is the optimal phase to observe the effects that compounds have on the cells.

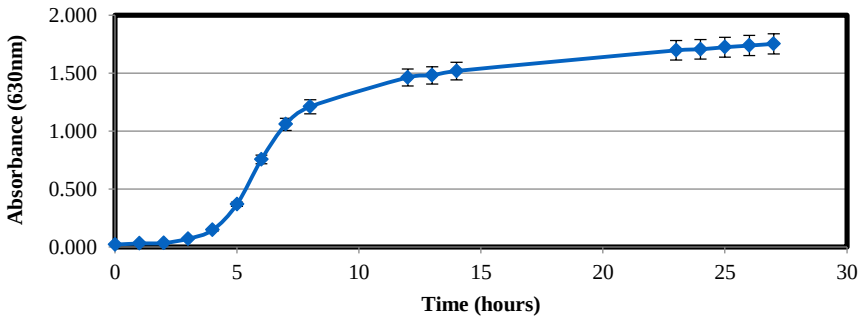


Figure 3.4: Growth curve of *E.coli* HB101
Error bars represent the standard deviation of the mean *(n=3)*

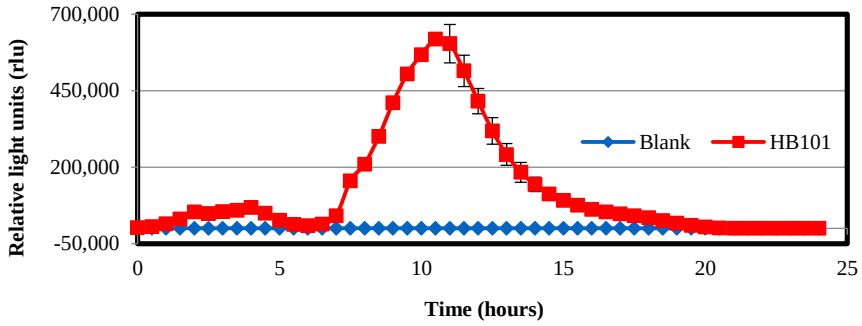


Figure 3.5: Luminescence curve of *E.coli* HB101
Error bars represent the standard deviation of the mean *(n=3)*

3.2.4 Lyophilised stock

Lyophilising or freeze drying stock helps to overcome the problem of time loss due to the long lag phase involved in culturing the biosensor. Also, it enables consistency and compounds to be tested against the same bell batch. Bacterial consortia were taken from active bioreactor treating spent MWF waste. Then, cells were grown to mid-exponential phase in LB broth. Cells were then centrifuged (Megafuge 11R, Thermo Scientific), re-suspended in *mist dessicans* (a mixture of 3 parts serum and 1 part broth with 7.5% glucose added) (Heckly, 1961), and immediately frozen at -80ºC. After 12 hours at -80ºC cells were lyophilised in an LTE cryopreservation unit (mini Lyotrap, LTE). Cryopreserved cells were then stored at -20ºC prior to use.

Figure 3.6: One mL freeze dry stocks being made in the mini Lyotrap machine

3.2.5 Preparation of test samples

3.2.5.1. Single compound screening

A dilution series was prepared for eighty test samples composed of components of all of the major classes of MWF components (supplied by BP-Castrol Ltd.) (see Appendix I). Individual compounds were diluted in water to 7 concentrations; unit v/v for all; 0.001%, 0.005% 0.01%, 0.05%, 0.1%, 0.5%, and 1.0%, respectively. These diluted samples were used for toxicity testing (see section 3.2.6) and degradability assessment (see section 3.2.8), where extra 11.28g/L M9 minimal growth media (Sigma) and 0.56g/L Magnesium Sulphate heptahydrate, $MgSO_4$ (Acrōs Organics) were added in the degradability assay.

Figure 3.7: Toxicity sample dilutions for the first 35 compounds

3.2.5.2. Combinatorial studies

As an example, codes were assigned for 2 compounds combination samples as indicated in Table 3.4 (see Appendix I and II for compound code and full compound combination Tables).

Table 3.4: Code labels for 2 compound combinations experiment

Code	Compound A	Compound B		Code	Compound A	Compound B
2.1	A12	B3		2.15	B9	B30
2.2	A12	B9		2.16	B9	C2
2.3	A12	B10		2.17	B9	C3
2.4	A12	B30		2.18	B9	D1
2.5	A12	C2		2.19	C3	D1
2.6	A12	C3		2.20	B10	B30
2.7	A12	D1		2.21	B10	C2
2.8	B3	B9		2.22	B10	C3
2.9	B3	B10		2.23	B10	D1
2.10	B3	B30		2.24	B30	C2
2.11	B3	C2		2.25	B30	C3
2.12	B3	C3		2.26	B30	D1
2.13	B3	D1		2.27	C2	C3
2.14	B9	B10		2.28	C2	D1

As with the single screening, 255 possible combinations (Equation 2 and Table 3.5) for 8 compounds (see Appendix II). Compounds were tested for toxicity, post-exposure recovery, and compound degradability test (see sections 3.2.6, 3.2.7, and 3.2.8). However, for toxicity and post-exposure recovery testing, the results were recorded after 15 minutes instead of 24 hours exposure. Acclimated bacterial community from an active bioreactor was used as an inoculum for post-exposure recovery in order to be comparable with degradability data.

Equation 2: Calculation for number of combinations (ignoring orders)

$$\binom{n}{k} = \frac{n!}{k!(n-k)!} = \frac{n(n-1)\cdots(n-k+1)}{1\cdot 2\cdots k},$$

n = total number of components; k = number of selected combinations

(Kreyszig *et al*., 2011)

Table 3.5: Number of possible combinations in each complex level (ignoring orders)

No. of compounds in the combination	No. of possible combinations
1	8
2	28
3	56
4	70
5	56
6	28
7	8
8	1
Total	**255**

3.2.5.3. Amine substitution

Forty-five possible combinations of new MWF formulation were tested for their biodegradability. Lowest optical density indicates the biological hardest combination in terms of biological resistance.

These samples were prepared by replacing A12 (Triethanolamine) with the following amines in Table 3.6 and other formulations were also tested (see Table 3.7). The example is shown in

Table 3.8. These amines were carefully selected in co-operation with BP-Castrol.

Table 3.6: Possible amines and concentration for compound A12, Triethanolamine, substitution

Amine	Concentration (%)
A1	15.0
A3	
A4	
A10	
A11	
A20	
A22	

Amine	Concentration (%)	Amine	Concentration (%)
A6	0.5	A10	14.5
A7	1.0	A10	14.0
A14	2.5	A10	12.5
A6	0.5	A12	14.5
A7	1.0	A12	14.0
A14	2.5	A12	12.5

Table 3.7: Chemical constituents in 6, 7, and 8 compound combinations in amine substitution testing

Compound Combination	Chemical Constituents
6	Replacement of B3 with B10 compound and absence of D1 compound
7	Replacement of B3 with B10 compound and D1 remain presence
8	Original formula; contained B3, B9, B10, B30, B31, B32, D1

Table 3.8: Example of sample preparation (original 8 compounds combination formula) for amine substitution.

Combination Code	Vol
A 8.1	ml
H2O	45.0
A1	15.0
B3	1.0
B9	2.5
B10	5.0
B30	1.0
B31	15.0
B32	15.0
D1	0.5

Combination Code	Vol
A 8.2	ml
H2O	45.0
A3	15.0
B3	1.0
B9	2.5
B10	5.0
B30	1.0
B31	15.0
B32	15.0
D1	0.5

Amine A12 was replaced by amine A1 in combination 8.1 and amine A3 in combination 8.2, remaining the rest of the constituents the same.

3.2.6 Toxicity test by biosensor

Plates were prepared by adding 180µL of sample compound dispensed into the 96 vials plate (Nunc brand). The inoculum was resuscitated by adding 2mL distilled water into the freeze dry stock of *E.coli* HB101 (see section 3.2.4) and left at 25ºC for 2 hours. The cells were washed in Phosphate Buffered Saline (PBS) (0.01M pH 7.4, Sigma) and re-suspended in water. They were then aliquoted 20µL into each well of the 96 wells plate. Relative light units were measured and recorded by microtitre plate spectrophotometer after 15 minutes of exposure to the test compounds. The test was done in triplicate for each of the eighty compounds at 7 dilutions and 255 combinations (see section 3.2.5.1 and section 3.2.5.2). Distilled water was used as control in this experiment.

3.2.7 Post-exposure recovery test (PER)

Miles & Misra method (Miles *et al*., 1938) is a standard plate counting method, in which instead of 0.1mL of solution being added to the whole plate, 20µL is spotted a small part of the plate. This miniaturised method enables up to eight spot and thus individual colony forming unit (CFU) per plate (Figure 3.8). Post-exposure recovery tests were undertaken employing the Miles & Misra (Miles *et al*., 1938) plate count method to determine colony counts after 15 minutes exposure to the test compounds. The aim of this experiment was to determine the recovery rate of the biosensor after having exposed to the test compound. Result can reflect positive or negative effects of individual chemical constituents relative to

the controls. If the counts were greater than the control, this indicates positive growth and the stimulatory impact of the tested compound. Zero growth indicates the lethally of the test compound to bacteria. After 15 minutes of exposure, 100µL of the aqueous solution of chemical and bacteria was taken from the 96 vials plate in toxicity test (see section 3.2.6), diluted 6 times and re-grown in a static incubator at 35ºC for 18 hours. The colonies were counted (Colony counter SC6, Stuart) and recorded. Distilled water was used as control in this experiment. The test was done in triplicate for both single screening and combinatorial studies to allow statistical testing of the data (see section 3.2.5.1 and section 3.2.5.2).

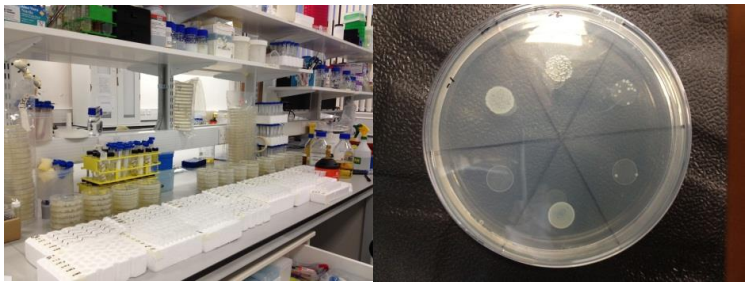


Figure 3.8: Dilution process for Miles & Misra plate count method (Miles *et al.*, 1938). 6 times dilution for each compound (left) and re-growing on LB agar plate (right)

3.2.8 Degradability test

Degradability assessments were performed using a MWF acclimated bacteria community, in this case taken from a long established lab-based bioreactor used for treating waste MWF. Bioreactor communities are diverse and actively growing and in this specific case adapted on MWFs (Madigan *et al.*, 2009; van der Gast *et al.*, 2004; van der Gast *et al.*, 2003a; van der Gast *et al.*, 2003b; Emmerling *et al.*, 2002). Cells were washed in 0.1M PBS before inoculation into the plate (Sterilin vitro). The optical density at 600nm was recorded every 30 minutes for 24 hours. The test was carried out in triplicate for eighty

compounds individually at 7 different concentrations and in compound combination (see section 3.2.5.1, 3.2.5.2, and 3.2.5.3).

Figure 3.9: The absorbance 96 vials plate employed for degradability test (left) and the luminescence 96 vials plate for toxicity test (right). Note that black plate required for luminescence testing was employed to exclude external light.

3.3 Results and discussions

3.3.1 Single screening experiment

The single screening results from the 80 generic compounds test provided key baseline information for the subsequent combinatorial studies of increasing complexity. The results were expressed in three ways; the toxicity (see section 3.3.1.1), lethality and relative recalcitrance of the test compounds (see section 3.3.1.2), and the growth of bacteria on each test compound (growth across 7 dilutions, see section 3.3.1.3). This is particularly valuable information to understand the toxicity and degradability of each individual compound for future MWF formulation development. From the summarised raw data (see section 3.3.1.4), the compounds were categorised into four main categories, namely, lethal, recalcitrant, inhibitory, and benign. Then particular interactions with co-compounds and exposed cells were tested in combinatorial studies (see section 3.3.2). Furthermore, the biodegradability of operationally exhausted MWF is examined in Chapter 4 in the context of the knowledge gained from single and combinatorial testing detailed above.

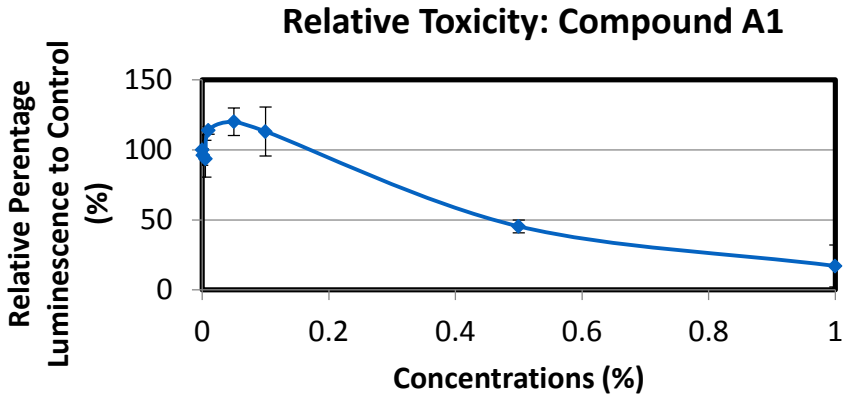
3.3.1.1. Toxicity testing for individual compounds

Bacterial biosensors such as *Vibrio fischeri*, recA, and *Escherichia coli* HB101 *lux* have been used extensively in various toxicity tests including acid sulphate soil in the sea, plant secondary compounds, and soil contaminants (Wallin *et al*., 2015; Chan *et al*., 2013; Maderova and Paton, 2013). Although, *Vibrio fischeri* seems to be a very popular choice as a biosensor, it may not be ecologically representative since it is a saltwater bacteria, thus testing requires solution to be adjusted with sodium chloride potentially changing the chemistry. Therefore, there has been an increased use of biosensor based to terrestrial bacteria such as *lux*-marked *E.coli, P. fluorescens*, and *P. putida* (Strachan *et al*., 2001). These tests are rapid and cost effective measures of toxicity for a range of environmental applications (Su *et al*., 2011; Lei *et al*., 2006; Belkin, 2003; D'Souza, 2001). Furthermore, bacterial bioassays can be used to interpret acute toxicity or chronic or mutagenic responses (Strachan *et al*., 2001). Toxicity work on MWFs has overwhelmingly focused on assessing risk to humans (Gordon, 2004; Cheng *et al*., 2005; Geier *et al*., 2003; Virji *et al*, 2000). These studies used higher organisms such as fish and rat as surrogates for humans in toxicity testing (Bai *et al*., 2013; van der Schalie *et al*., 2001; Talmage, 1994). For example, caprylic acid, an MWF component, was found to be non-toxic in Figure 3.12 in agreement with the literature, for rats (Bai *et al*., 2013). However, the use of higher organisms is expensive, emotive, time consuming, and may not reflect the responses of bacteria in the system in this research. Ribo and Rogers (1990) have used the Microtox TM luminescence-based microbial biosensors and have proved that microbial test has significant advantages over the use of higher organisms to measure the toxicity. Therefore, the responses of a bacterial biosensor have been used to assess toxicity in our bacterial-based experiments during the degradation process for individual and combined MWF components. Biocides for example, we found to be toxic in previous studies see Figure

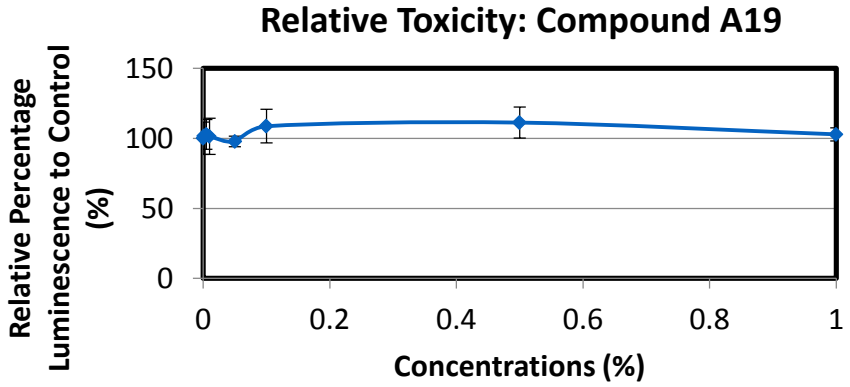
3.12 and Table 3.10. In this study, there was no light detected after exposure to biocide samples (Rigol *et al*., 2004).

Moreover, previous researchers have used bacterial biosensors to assess the toxicity of compounds such as benzene and methyl *tert*-butyl ether (Roslev *et al.,* 2015; Boyd *et al.,* 1997). These were single parameter tests using light output only as the measure of toxicity. Recent work from our group has extended on this approach by including post-exposure recovery test (Chan *et al.,* 2013). Here in this study we have further extended on this using *E.coli* HB101 bioluminescent in combination with post-exposure recovery, and acclimated bacterial growth assays. This provides a deeper multiple parameters insight into the level of toxicity of the individual compounds and can distinguish between transient inhibitory effects or lethally toxic insults.

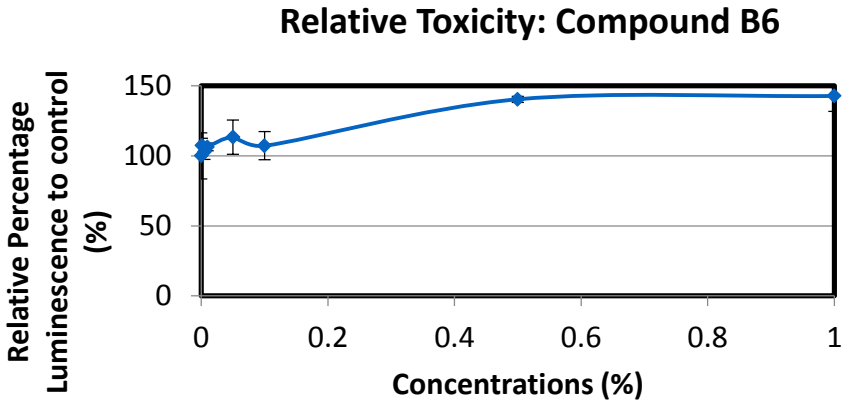
The figures below are examples of luminescence curves produced from single toxicity screening. The graph represents the effect of the compounds on the *E.coli* HB101 on a gradient from 0 – 1 % v/v concentration. The results are expressed as percentage relative light units in order to be able to account for slight variation in biosensor light output between batches and allow comparisons across plates.



(a)



(b)



(c)

Figure 3. 10: Some examples of the biosensor response to selected MWF components (a) Compound A1 shows a typical toxic response. Light output is reduced on exposure to the test compound. (b) Compound A19 shows a typical non-toxic response. (c) Compound B6 shows a stimulatory response. The tests were done in triplicate. Error bars represent the standard deviation of the mean *(n=3)*

Much research has shown that there was a dose response relationship between the organic test compound concentration and the toxicity responses. For example, it was reported as the concentration of herbicides increased, the light emitted by the biosensor declined (Strachan *et al*., 2001).

Note that Figure 3. 10 represents the responses along a concentration gradient based on 7 concentrations. To summarise and allow comparisons to be made with growth and post-exposure recovery results, data for 1% v/v concentrations were transformed to percentages (see Figure 3.12).

The results represented in Figure 3.12 showed that approximately 20% of eighty compounds were readily degradable with low toxicity, but the majority of the components analysed were toxic or inhibitory to exposed bacterial cells. Toxic compounds have historically been specifically selected to make the MWF formulation resistant to biodegradation whilst in use on the machine. However, this is not compatible with end-of-life biotreatment an increasingly favoured sustainable option for disposal.

3.3.1.2. Post - exposure recovery (PER)

Post-Exposure Recovery experiments were used to determine the recovery rate of the biosensor after exposure to the test compound at 1% v/v concentration. It can be seen from section 3.3.1.4 and Figure 3.12 that the majority of compounds tested were lethally toxic and that the biosensor did not recover after the exposure. This assay allowed inhibitory and lethal effects to be distinguished.

On average, bacterial counts in the water control were 3.35 x 10^6 CFU/ml. Many compounds, such as C1, were found to be non-lethal as the *E.coli* could be re-grown after exposure to the chemical component. Conversely, compound C20 and C22 were found to be lethal *i.e*. toxic with no post-exposure recovery. Similar to toxicity test, recorded port-exposure recovery was converted to percentages in order to make comparisons with toxicity and growth results.

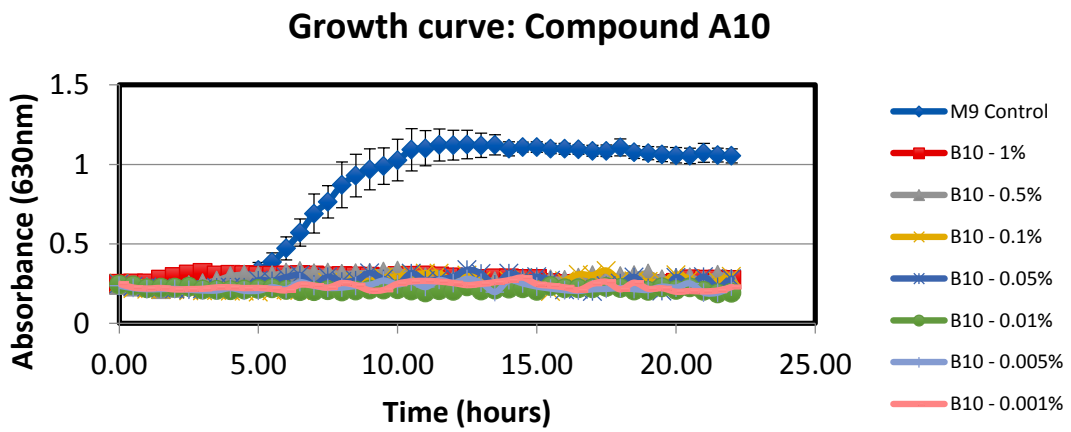
Table 3.9: Table of recovery rate of *E.coli* exposed to the individual generic MWFs components in Syntilo 9913. The experiment was carried out in triplicate. The table shows a few examples.

Compounds	**Average Colony Forming Unit (CFU)**	**Standard Deviation (SD)**	**Percentage of control (%)**
H2O	3.35E+06	1.91E+04	100
C1	4.44E+06	1.28E+04	132.5
C2	1.73E+06	9.70E+03	51.66
C3	2.44E+06	1.11E+04	72.89
C4	1.93E+06	1.14E+04	57.46
C15	2.70E+06	4.68E+03	80.6
C16	2.12E+06	6.03E+03	63.18
C18	2.94E+05	1.87E+02	8.79
C20	0.00E+00	0.00E+00	0
C21	1.06E+06	9.72E+03	31.57
C22	0.00E+00	0.00E+00	0

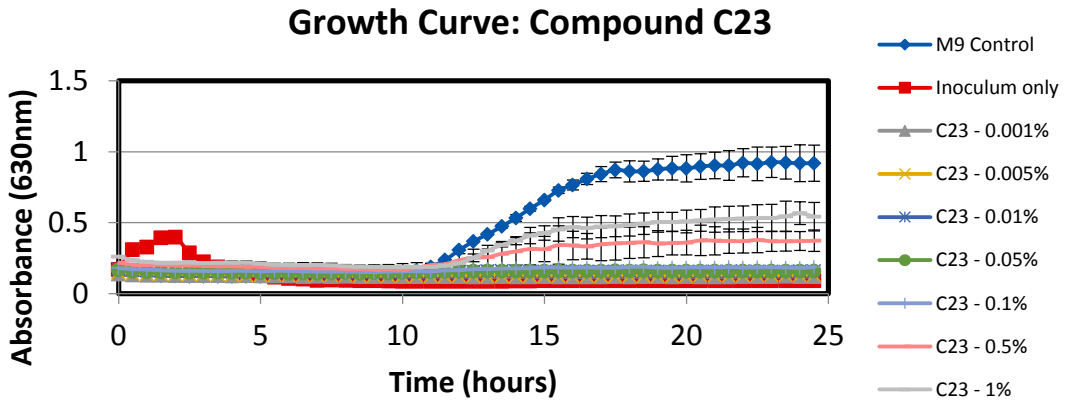
Data represented in Figure 3.12 show that after exposure cells were only recovered for approximately 12.5% of eighty compounds tested. By combining the PER and results from the toxicity test, it can be seen that 9% of the compounds were inhibitory and toxic since no instances of post exposure recovery were recorded. The proportion of inhibitory and lethal compounds was approximately 1:5.

3.3.1.3. Single degradability test

Degradability testing provided insight into whether a compound could be utilised as a sole carbon source by the bacteria inoculated into the plate. Growth was determined by optical density (OD) (see Figure 3.11). Increased OD equated to growth and increase of cells numbers. A lack of growth could be interpreted in two ways. 1: the compound was toxic and had killed the inoculum, or 2: the compound was not bioavailable and so did not support bacterial proliferation. Constructing growth curves at different concentrations provide some insight into the possible mechanism. At low compound concentration, bacterial growth may be limited by carbon availability, for example; bacteria grew to a greater density at 1% than 0.5% concentrations of compound C23, suggesting carbon limitation in the 0.5% concentration. Growth curves were constructed from triplicated samples. In contrast, less growth at high compound concentrations indicated that the compound was toxic as there is no growth in the test carbon source (Figure 3.11). Growth curves showing the potential of a carbon source to sustain microbial growth are presented in Figure 3.12.



(a)



(b)

Figure 3.11: Examples of individual growth curves at 7 concentrations. (a) Bacteria could not grow in compound A10; (b) bacteria grew to a greater density at 1% than 0.5% concentrations of compound C23, suggesting carbon limitation in the 0.5% concentration. Growth tests were done in triplicate. Error bars represent the standard deviation of the mean *(n=3)*

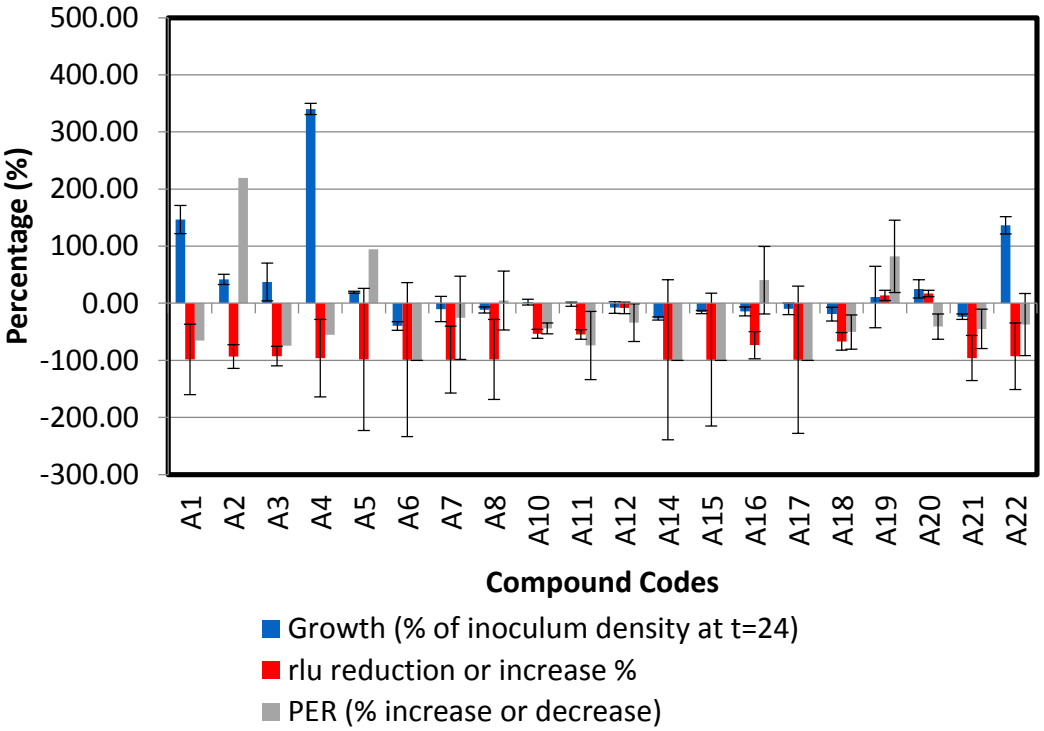
Figure 3.11 shows examples of the growth responses in a concentration gradient of the test chemicals. To summarise the data for all compounds and allow comparisons to be made with toxicity and post-exposure recovery results, data for 1% concentrations were transformed to percentages, see Figure 3.12.

From the literature, it has been reported that bacterial consortia are more effective in degrading test compounds compared to single strains. The rationale could be that one of the strains can degrade the compound to certain product, and another strain(s) continue to degrade to aid the complete degradation. The evidence was tested with Methyl t-butyl ether (MTBE) (Mo *et. al*., 1997).

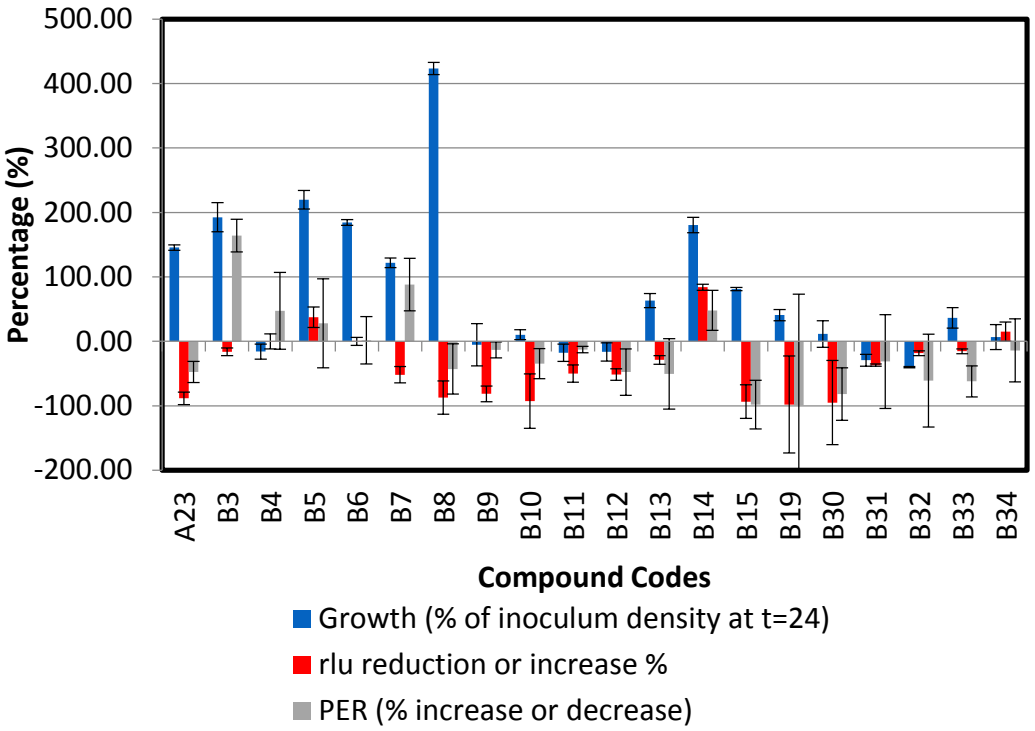
There have been many studies in which growth in sole carbon sources has been used as a measure of the biodegradability of the test compound (Mo *et al.,* 1997; Dong *et al.,* 2015). The normal S curve is produced when the compound is biodegradable as it shows the growth of bacteria community. For example, degradation of aliphatic monoamine such as methylamine has previously been reported (Eady and Large, 1968). However, some chemical components are non-biodegradable. For example, TEA was found to be non-biodegradable constituent of the MWF wastewater (Seo *et al*., 2007), which is in agreement with the observation in this study.

3.3.1.4. Summary of single compound screening

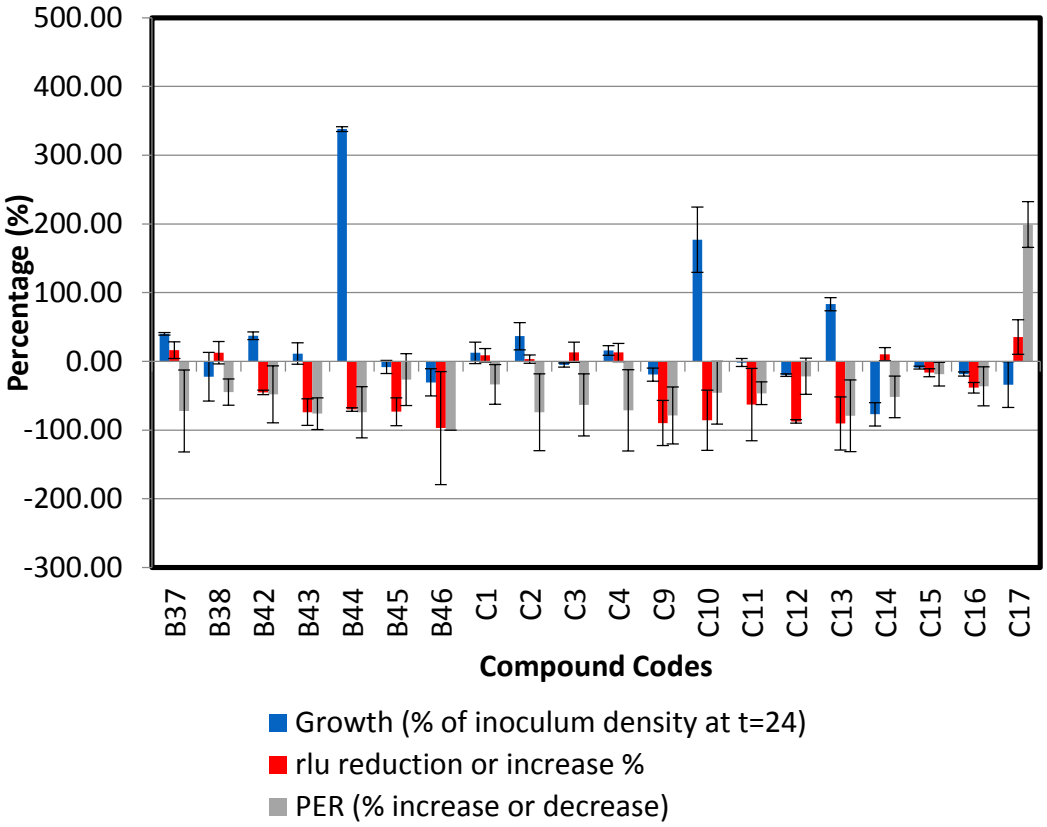
The summary of results is represented as the graphs below. This shows the growth, toxicity, post-exposure recovery relative to the water control for eighty single compounds. The combination of these three parameters represents a novel approach for obtaining deeper insight into the toxicity profile of individual MWF components.


500.00
400.00
300.00
200.00
100.00
0.00
-100.00
-200.00
-300.00
Percentage (%)
A1
A2
A3
A4
A5
A6
A7
A8
A10
A11
A12
A14
A15
A16
A17
A18
A19
A20
A21
A22
Compound Codes
Growth (% of inoculum density at t=24)
rlu reduction or increase %
PER (% increase or decrease)

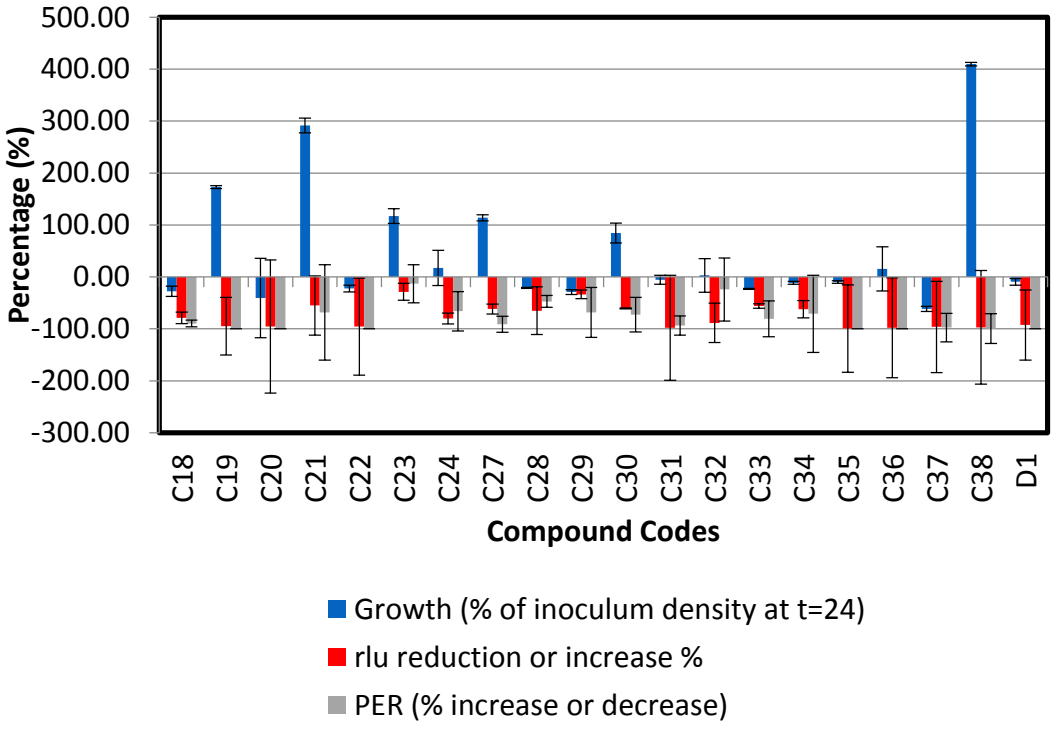
(a)


500.00
400.00
300.00
200.00
100.00
0.00
-100.00
-200.00
Percentage (%)
A23
B3
B4
B5
B6
B7
B8
B9
B10
B11
B12
B13
B14
B15
B19
B30
B31
B32
B33
B34
Compound Codes
Growth (% of inoculum density at t=24)
rlu reduction or increase %
PER (% increase or decrease)

(b)



(c)



(d)

Figure 3.12: Summary of single compound screening of eighty compounds based on growth community, toxicity, and post-exposure recovery. Tests were done in triplicate. Error bars represent the standard deviation of the mean *(n=3)*

From this, it can be seen that there was a mixture of responses, some tested compounds stimulated growth and activity whilst others were toxic and suppressed activity or specifically were lethally toxic (45%), benign (3.75%), inhibitory (6.25%), recalcitrant (1.25%), and toxic to *E.coli* HB101 specifically (30%). An example of the class of response to chemical exposure is described below:

Compound A15 was classified to be "lethally toxic"; the results from the three graphs (Figure 3.*12*) indicate that both biosensor and acclimated mix community were killed by the test sample.

Compound A2 was classified to be "inhibitory", after 15 minutes of exposure the biosensor did not give any response. However, it was able to recover when the cells were transferred to a culture plate containing none of test compound.

Compound C17 was classified to be "recalcitrant"; the growth of the acclimated mix community was suppressed, whereas the biosensor gave a positive response.

Compound B5 was classified to be "benign"; both biosensor and acclimated mix community were not damaged by the test sample.

Compound A4 was classified as "toxic to *E.coli* HB101" in that it generated negative relative light emission and post-exposure recovery indicated that it was lethal to the biosensor, however, there is a large peak for growth in the acclimated mix community. This is due in part to the acclimation of the mixed community to MWFs and also probably due to greater resilience from the community because of the functional diversity and redundancy of its members.

Bacterial responses to MWFs constituents were summarised in Table 3.10. This represented key baseline information for the subsequent *in silico* predictions and combinatorial experiments in the laboratory. In preliminary work, there were several observations of counter-intuitive responses to compound combinations. It can be seen that some combinations of two or more compounds changed the nature of the properties of the individual compounds (section 3.3.2 and Figure 3.13). For example, the combination in which two biocides and an amine were expected to be very toxic, since as individually they proved to be very toxic. However, addition of extra biocide to the two biocides and an amine cocktail was found to neutralise the mix and altered its classification from toxic to moderately benign, although 3 individual biocides on its own were very toxic.

Table 3.10: Classification to compounds' properties from single compound screening

Classification	**Compound code**					
Lethally Toxic to mixed community	A6	A14	B11	C9	C20	C33
	A7	A15	B12	C11	C22	C34
	A8	A17	B31	C12	C28	C35
	A10	A18	B32	C15	C29	C36
	A11	A21	B45	C16	C31	C37
	A12	B9	B46	C18	C32	D1
Inhibitory	A2	A5	A16	B3	B7	
Recalcitrant	C17					
Benign	A19	B5	B14			
Toxic to *E.coli* HB101 specifically	A1	A23	B15	B42	C13	C24
	A3	B8	B19	B43	C19	C27
	A4	B10	B30	B44	C21	C30
	A22	B13	B33	C10	C23	C38
Equivocal classification-further categories need to be delineated	A20	B6	B37	C1	C3	C14
	B4	B34	B38	C2	C4	

3.3.2 Combinatorial study

The screen described in this report was designed to identify these unexpected interactions and to inform the exploitation of their combined effects in future MWF formulations for "hardening" or "softening" purposes. An understanding of the effects of interactions between specific compound combinations on bacterial growth provided the basis for designing a new generation of MWFs with more predictable performance in terms of interaction of microbial cells. This includes resistance to biodeterioration of in-use and biological treatment end-of-life.

Previous preliminary testing of bacterial growth and toxic responses to combinations of biocides revealed that two or more toxic compounds neutralised the toxic effects of the individual compounds, so that the combination was much less toxic than when present as the single compound. Thus, in some cases observed and predicted (from the single compound) responses diverged (see Figure 3.13).

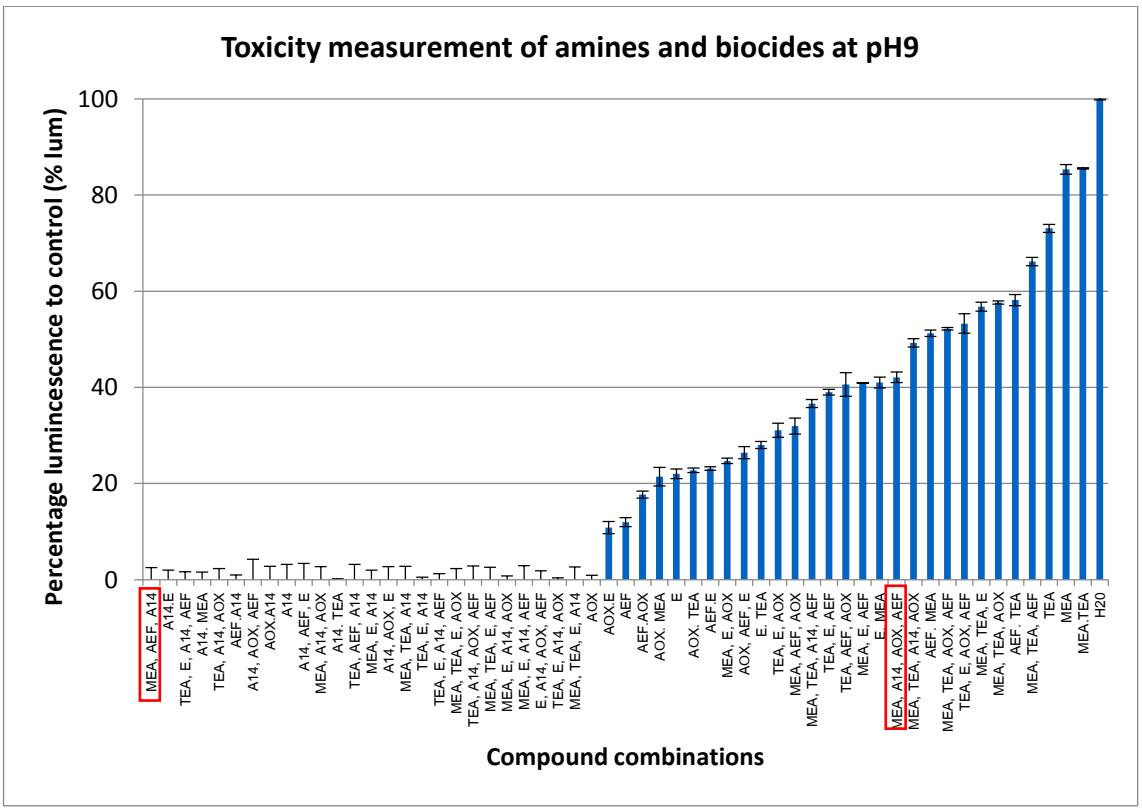


Figure 3.13: Toxicity assessments of chemical combinations of increasing complexity (2, 3, and 4 compounds) composed of the following components: Methanolamine (MEA), Triethanolamine (TEA), Emulsifier (E), Acticide 14 biocide (A14), Acticide EF biocide (AEF), and Acticide OX biocide (AOX). This shows the responses in percentile and was done in triplicates. Red boxes indicate the example where an additional biocide, AOX, was added to the toxic mixture and convert to benign-mixture. The tests were done in triplicate. Error bars represent the standard deviation of the mean *(n=3)*

Figure 3.13, provides examples of combinatorial studies which overall suggests that 48% of combinations were very toxic. Interestingly, it can be seen that a combination of Methanolamine (MEA), Acticide 14 biocide (A14), and Acticide EF biocide (AEF) was the most toxic in terms of light emitted by biosensor *E.coli* HB101 (left end of the graph), but once Acticide OX (AOX) was added into the mixture; MEA, AOX, A14, and AEF, the resultant was found to be in top 20% most benign combinations.

There is a considerable body of research in to the toxicity and effectiveness of biocide. Usually bacteria are killed in less than 12 hours in cutting fluids containing biocides. Although, biocide have been demonstrated to inhibit microbial growth in the MWF (Chazal, 1995) at lower concentration biocides have been shown to sustain growth of bacteria such as *Pseudomonas putida* and *Psuedomonas fluoresens* (Chazal, 1995).

Current legislation (EU Directive 67/548/EEC) requires that toxicity and environmental assessment is based on the single compounds and material safety data sheet "unless there is evidence to the contrary, authorities generally enforce regulations that assume that acceptable concentrations for pollutants can be treated independently, even when they are present in mixtures (Walker *et al*., 1996; Strachan *et al*., 2001)". This emphasises the importance of individual compound screening. Furthermore, a key point raised in ecotoxicological legislation is that the particular trophic level of the organisms is of great importance, as certain pollutants interact specifically with organisms of a particular trophic level. This is not the case in a mixed system such as environmental systems. (Strachan *et. al.,* 2001). As the results in this Thesis demonstrate, interactions between components in mixtures mean that responses to complex formulations do not necessarily correlate with the single compound assays and so an accurate picture of toxicity is not gained (see sections 3.1.2, 3.2.5.2, and 3.3.2). Hence, the toxicology of compound mixtures are not predictive and often counter intuitive when based on single compound responses (see Figure 3.15).

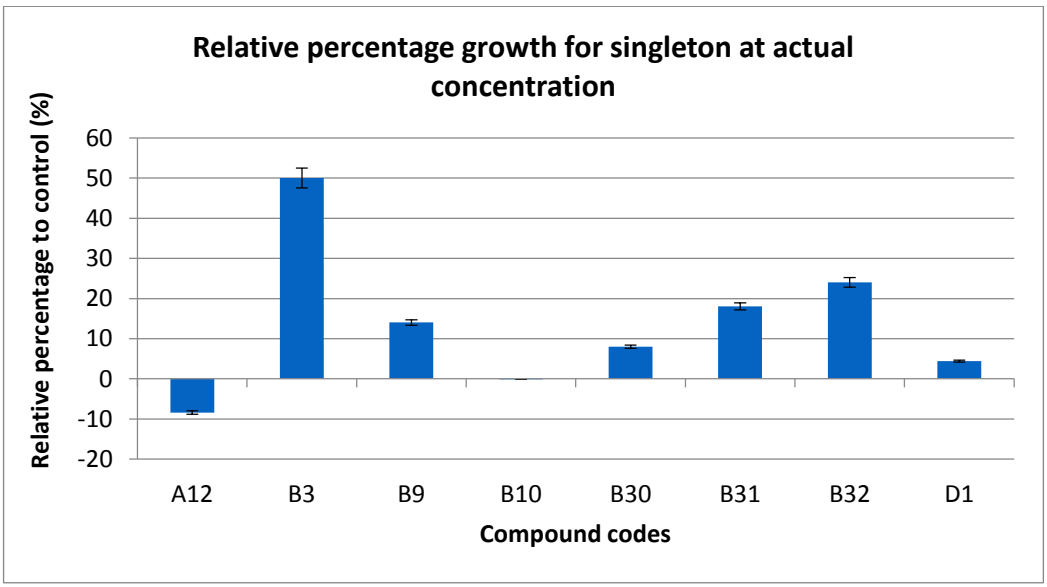


Figure 3.14: Relative growth expressed as the percentage of the control of individual component compounds present in Syntilo 9913 at actual manufactures concentrations. Tests were done in triplicate. Error bars represent the standard deviation of the mean *(n=3)*

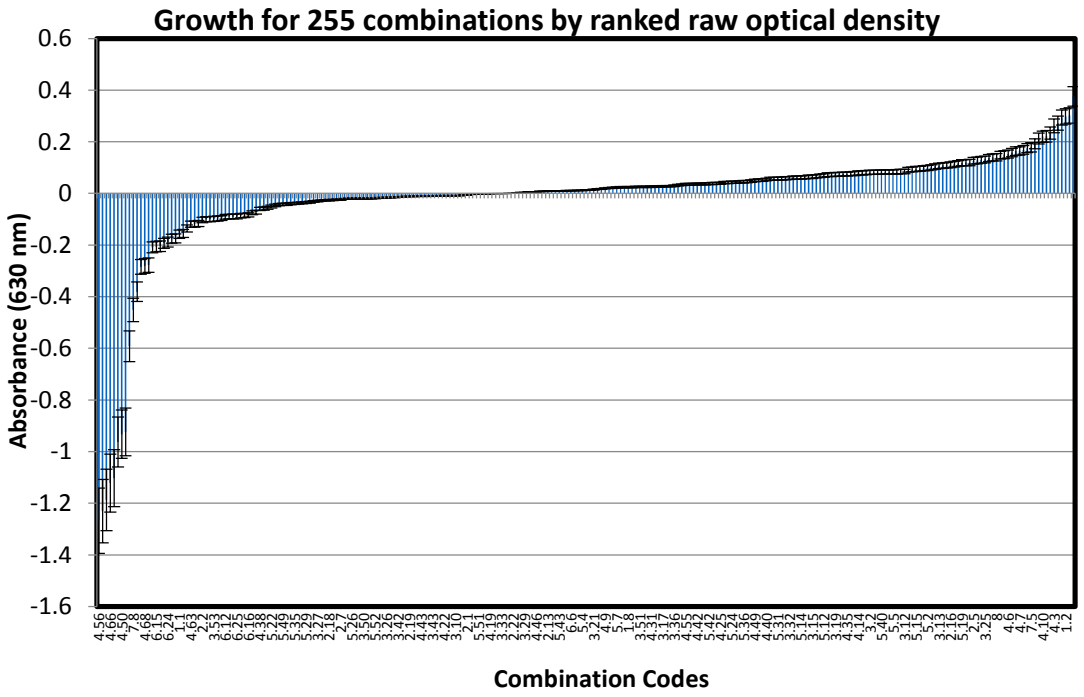
To enable comparisons between the compounds tested, data for the chemical combination assessments were expressed as percentage of control for toxicity, post-exposure recovery, and as growth in the same way as the single compound data (Figure 3.12). Data for single compounds were then used to predict the responses of combined compounds. To achieve this, eight constituents of Syntilo 9913 MWFs were screened individually at the actual concentration used in the commercial formulation (Table 3.2). To calculate the predicted growth value, the values of 2 compounds from single screen (Figure 3.16 and Table 3.4) were added, and then halved. The same calculation was performed for toxicity, growth, and post-exposure recovery assessments. Experimental data was compared with computed predictions for each of the toxicity, growth, and post-exposure recovery to identify compounds that produced responses that deviated strongly from the predictive model. This was also done for 3, 4, 5, 6, 7, and 8 compounds combinations. A model was built based on growth using single compound data and assuming no interaction (see Figure 3.15a). Note

the contrast with Figure 3.15b, this indicates that interactions occur between MWF components since 3.15b assumes no interaction.

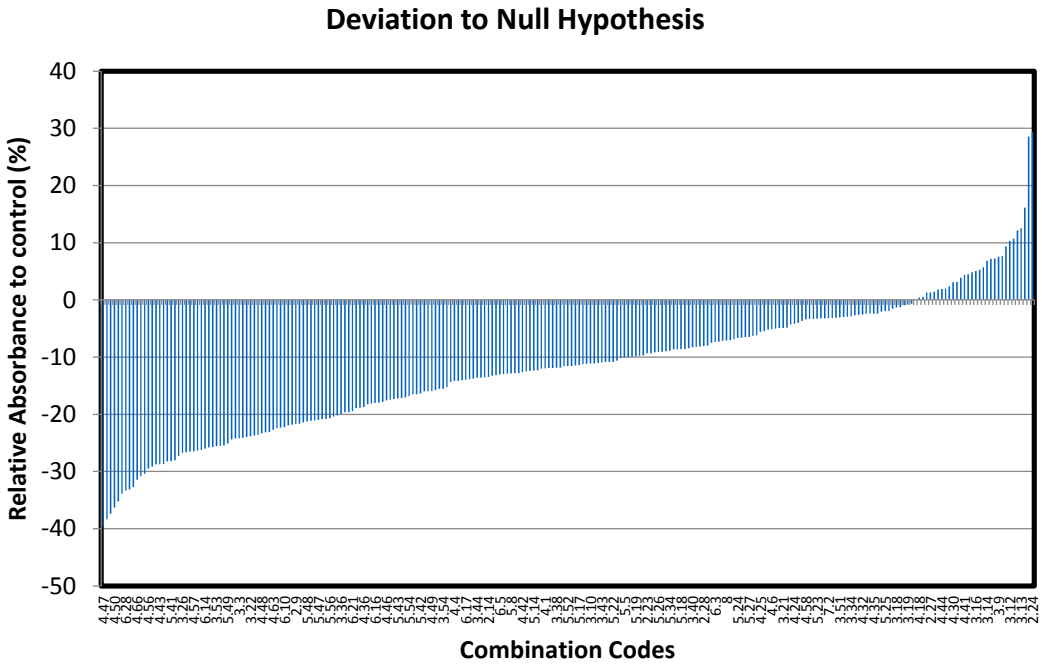
Equation 3: Deviation from Null Hypothesis = Relative % Growth – Predicted % Growth

From the study, it was found that the mixture of A12, B9, and D1, and mixture A12, B9, B10, B32 did not interact, but the other 99.2% of compounds tested were found interact. Therefore, simple additive model is not predictive of MWF component interactions.

Although, several studies suggest that the data for individual component can be used to predict the toxicity of the mixture as an independent source of data (Altenburger *et al*., 2000; Backhaus *et al*., 2000). However, early work in this project, the finding indicated that chemical interactions in mixtures can produce counter intuitive results that diverge from the actual observed toxic behaviour, for example two or more toxic compounds can combine to be benign and *vice versa* (*i.e* responses are not simply additive). This necessitates the combinatorial approach adopted in section 3.1.2 of this thesis. For example, the biocide work showed in Figure 3.13.



(a)



(b)

Figure 3.15: The differences in growth and predicted results: a) growth data b) deviation from null hypothesis. Error bars represent the standard deviation of the mean *(n=3)*

In one study, the majority of toxicity responses to multiple organics showed that the interactions were not additive but have antagonistic and often synergistic relationships (Strachan *et al*., 2001; Fernández-Alba *et al*., 2002; Preston *et al.*, 2000). However, the result from this study was not in agreement with findings with previous research, which suggested that the biological responses would be constant regardless of any interaction and they tended to behave independently (Stratton, 1989; Backhaus *et al.*, 2000). Strachan *et al*. (2001), Fernández-Alba *et al*. (2002), and Preston *et al*. (2000) concluded that the responses were too complex to be reflected by a simple additive model. Similarly, Mo *et al.* (1997) found that t-butanol, butyl formate, isopropanol, acetone and pyruvate were added to Basal Salt Medium in combination with Methyl t-butyl ether (MTBE), a reduction in the degradation of MTBE by *Methylobacterium mesophilicum* and *Rhodococcus sp.* occurred, as compared with the control. Work has been directed to accessing the biocide effect but less forcus has been on reporting antagonistic interactions whereby biocidal effects are neutralised (Figure 3.13). This suggests that simple additive or subtractive application of single compound data would be too simplistic to be predictive and that it requires more sophisticated analysis involving multiple parameters to construct a predictive model.

The data gathered in this study describe the bacterial metabolic response to exposure to MWF components and formulations. These responses must be the result of interaction at the molecular level involving reactive sites on the MWF component and the bacterial cell. Thus Structure Activity Relationships (SAR) in conjunction with bioreporter responses may provide insight into which chemical groups are interacting with cell molecules. SARs seek to describe and correlate physiological responses to specific chemical structures. Correlations may be qualitative (simple SAR) or quantitative (quantitative SAR, or QSAR)" (Organisation for Economic Co-operation and Development, 2015). For example, Huang *et al.* (2003) used an acute toxicity test using tadpoles as the bioreporter for benzene and

benzene derivatives. Chemical properties examined were charge, electron potential and molecule size. The model was built to predict toxicity that integrated the biological and chemical data. Bundy *et al.* (2001) employed *lux*-marked whole-cell microbial biosensor to test toxicity and biotransformation of a series of aromatic heterocyclic and hydrocarbon compounds. Chemical/Physical properties were predicted accurately from the modelled that was built from the data gained. Much research has applied SAR approach for make predictions on the relationship between chemical structure on various properties such as the biodegradability of organic compounds, alcohols, ketone, and alicyclic (Damborský, 1996; Vaishnav *et al*., 1987; Parsons and Govers, 1990), and sensitisation potential of MWF components (Anderson *et al.*, 2009). Anderson *et al.* (2009) reported that TEA and 4-chloro-3-methylphenol (CMP) were predicted to be positive for skin sensitisation on two different programs, TOPKAT and Derek, respectively. In collaboration with chemists and biologists, more precise prediction can be made toward bacterial activity and thus, degradability of the chemical interaction in the complex mixture.

Combining biological and chemical data there is a considerable opportunity to improve the understanding and find key chemical switches controlling the degradation potential of new MWF formulations. Moreover, the availability of such information should put formulation chemists in a much better position to design new MWF formulations that are resistant to biodeterioration when they are in use, but make them condusive to biotreatment, end-of-life.

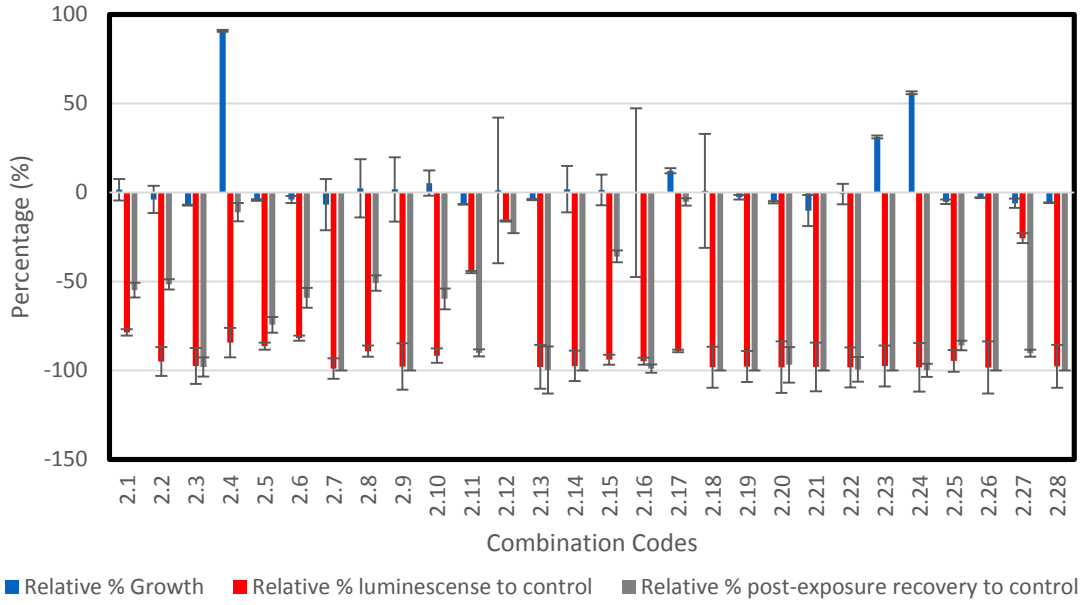


Figure 3.16: Summary of two compound combinations showing relative growth relative to control, different percentage relative light units percentage post-exposure-recovery (PER) to control. Tests were in triplicated. Error bars represent the standard deviation of the mean *(n=3)*

Figure 3.16 represents the relative degradability of the 2 compound combinations employing MWF-acclimated mix bacterial community. Often compounds, in combination, were observed to interact to produce responses that were not representative of the single compounds, being either more or less toxic than was predicted from single compound analysis. Combination 2.4 which consisted of A12 and B30, for example, was predicted, based on the response of the individual components, to be biodegradable. As can be seen in Figure 3.14, A12 was very resistant to biodegradation but B30 was moderately biodegradable. However, when present in combination the mixture stimulated growth of the acclimated community. The reason for the detected growth may have been the accumulated increase in carbon source when the two chemical constituents were combined.

A consideration for this experimental approach was that the predicted response for compound combinations was based on halving the concentration for each compound in a two compound combination (1/3 for 3 compound combinations); this could have potentially lead to the individual compounds being present below effective threshold levels. However, the majority of compounds produced linear and not threshold responses, giving support that the data set should produce reliable information. Another potential experimental variable was the use of an undefined bacterial consortium for growth experiments. Bioreactor communities are diverse, their composition are temporally variable, and also contains many uncultivable species making culture based inocula non-representative of an *in situ* community (van der Gast *et al.*, 2004, 2003a, 2003b).

The approach used here was based on a lyophilised (freeze dried preservation) community from an active MWF bioreactor. This is a bacterial community, which had long term exposure and presumably adapted to the toxic impact of MWF was employed as an indicator of toxic stress to the MWF constituents, as single entities and as mixtures. A bacterial community from an active bioreactor was also lyophilised as detailed in section 3.2.4. This assumes that the bacterial diversity and functional redundancy from the acclimated active bioreactor community was sufficient adapted that biodegradation would occur, despite some possible loss of viability during lyophilisation. All experiments were carried out from a single batch of stock to avoid founder effects leading to different community composition upon resuscitation and enabling comparisons between samples.

3.3.2.1. Summary of combination study results

The degradability testing, optical density data (growth) for 255 chemical combinations were measured and ranked as shown in Figure 3.17. Principle Component Analysis (PCA) was also undertaken in order to investigate key component interactions (Figure 3.18), however, all compounds were found to cluster together making it impossible to distinguish responses and therefore key components. To address this, a matrix was developed that revealed the softening or hardening characteristics of all the tested compounds (Figure 3.19).

Note that the percentile data was not very reliable since the transformation distorted the quantitative relationships and providing some misleading information; therefore raw optical density data was used to calculate the absolute growth as in Equation 4.

Equation 4: Calculation of absolute growth

$$\textbf{Absolute growth} = OD_{T24} - OD_{T0} - OD_{inoculum}$$

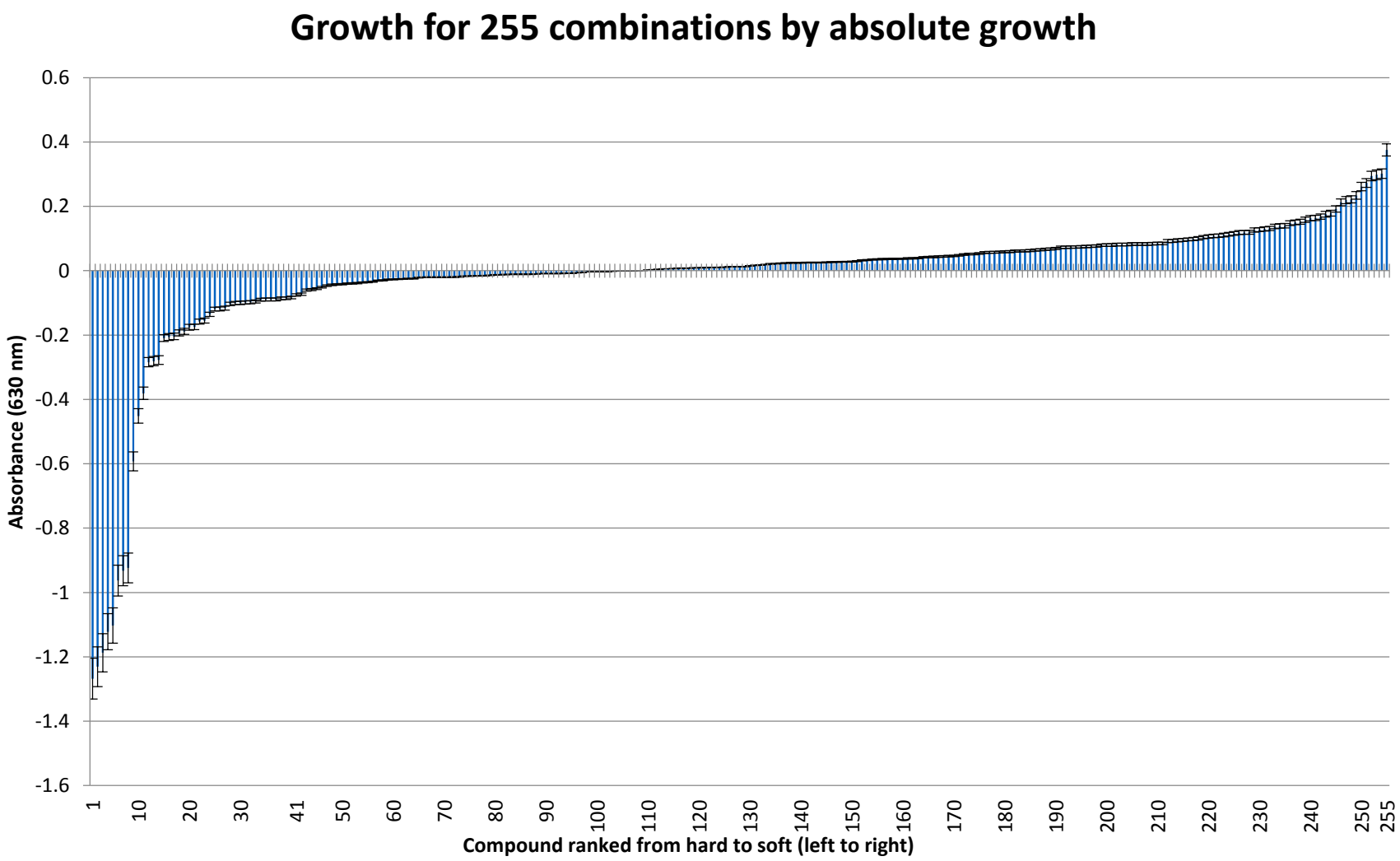


Figure 3.17: Absolute growth for 255 combinations ranked from the lowest value to the highest value. Tests were done in triplicate. Error bars represent the standard deviation of the mean *(n=3).* See Appendix 4 for complete compounds ranked by absolute growth.

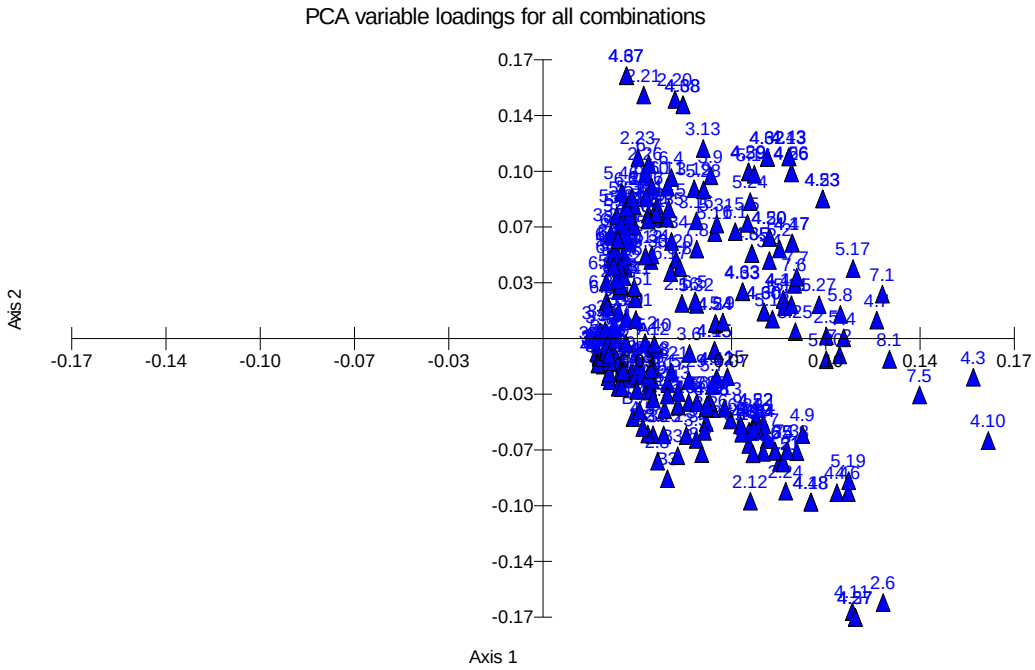


Figure 3.18: Principle Component Analysis (PCA) for absolute growth, toxicity, and post-exposure recovery of 255

Principal Component Analysis (PCA) is a very effective method for analysing multivariate data. This was employed to calculate how each variable influences each data point. However, in this case as is represented in Figure 3.18, the compounds tested clustered together and so was ineffective at detecting any useful trends that would help design better MWF or help in the next phase of the biodegradation study. Hence, an alternative matrix was developed as represented in Figure 3.19.

top 20 "soft“

Soft Combinations								
Combination Code	A12	B3	B9	B10	B30	B31	B32	D1
2.12		■					■	
2.6	■						■	
2.24					■		■	
4.11	■	■			■		■	
4.3	■	■	■			■		
2.8		■	■					
7.1	■	■	■	■	■	■	■	
4.10	■	■			■	■		
4.23	■		■			■	■	
3.5	■	■					■	
7.5	■	■	■		■	■	■	■
4.27	■			■	■		■	
4.21	■		■		■		■	
4.7	■	■		■		■		
7.2	■	■	■	■	■	■		■
4.6	■	■		■	■			
3.34		■				■	■	
5.20	■	■				■	■	■
8	■	■	■	■	■	■	■	■
5.16	■	■		■			■	■
	16	**15**	**8**	**7**	**10**	**10**	**14**	**5**

top 20 "hard"

Hard Combinations								
Combination Code	A12	B3	B9	B10	B30	B31	B32	D1
4.56			■	■	■	■		
4.67				■	■	■		■
4.47		■		■	■		■	
4.66				■	■	■	■	
4.69				■		■	■	■
4.60			■	■		■		■
4.50		■		■		■		■
4.59			■	■		■	■	
6.28			■	■	■	■	■	■
7.8		■	■	■	■	■	■	■
6.27		■		■	■	■	■	■
4.51		■		■			■	■
4.68				■	■		■	■
4.57			■	■	■		■	
2.4	■				■			
6.15	■	■			■	■	■	■
3.28		■		■		■		
5.28	■		■		■	■		■
6.24		■	■	■	■		■	■
6.19	■		■	■		■	■	■
	4	**8**	**9**	**17**	**13**	**14**	**12**	**13**

Figure 3.19: Top 20 most degradable (green) and least degradable (red) matrix from 255 possible combinations, which determined by yield of cells. The coloured boxes show the existence of the compound in the combination. (See Appendix I and II for compound code)

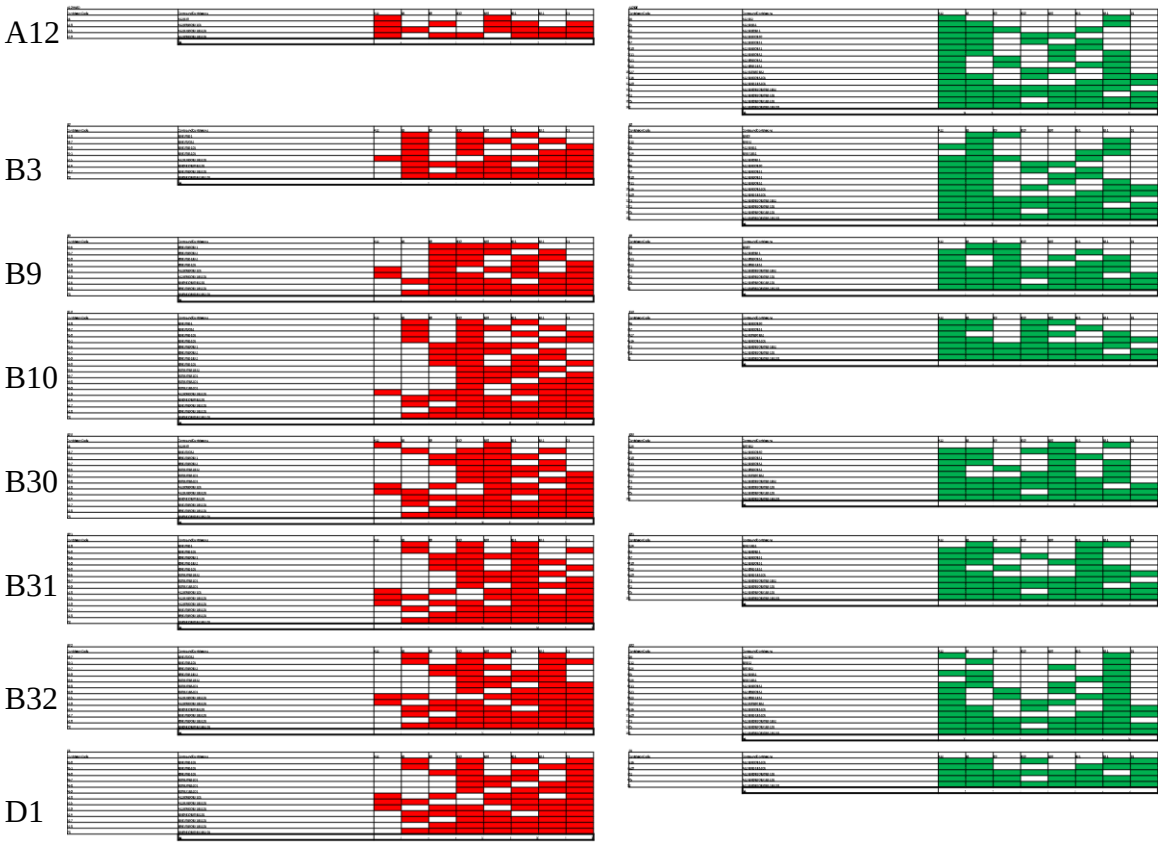


Figure 3.20: Trend of softening/hardening/intermediate key compounds. Red represents top 20 least biodegradable from 255 possible combinations and green represents top 20 most biodegradable combinations. For example, mixtures that compose of compound A12 are listed and split between weather that combination is in top or bottom 20 in terms of degradation and weigh whether the compound exist in red or green more. D1 and B10 are predominantly “hardening”, and A12 and B3 are predominantly degradable.

Matrix in Figure 3.19 shows the top twenty most degradable (green) and least degradable (red) chemical constituent combinations from ranked 255 combination according to the absolute growth of an acclimated bacterial community in 24 hours. Further matrix were developed (Figure 3.20) by creating matrix for each compound; A12, B3, B9, B10, B30, B31, B32, and D1; in order to classified whether the compound acted as a predominantly “softening” or “hardening” or “intermediate” compound. From Figure 3.19, it can be seen

that seven compound combinations, namely 7.8 composing of compound B3, B9, B10, B30, B31, B32, and D1, were in the top twenty most recalcitrant chemical combinations, however, when an eighth compound was added (A12 or Triethanolamine), which would provide a complete formulation composition for Syntilo 9913, the combination resulted in a product Syntilo 9913 that was far more readily degradable compare to combination 7.8. Thus, A12 (Triethanolamine) was identified to be the key determinant in terms of stimulating biodegradation. This was confirmed in Figure 3.20 that shows that compound A12 (Triethanolamine) and B3 (an acid, Corfree M1) were predominantly softening compounds. The results also suggest that the chemical structure of the compound could have the effect on the degree of the biodeterioration. For example, Triethanolamine (A12) has 3 hydroxyl groups, which makes the compound hydrophilic, which is believed to facilitate transfer across the cell membrane so stimulating biodeterioration and biodegradation. It can be broken down into smaller compounds to formaldehyde and ammonia as the end products, and complete biodegradation has been reported (Kim *et al.*, 2001). This is not ideal when it is in operation, as this shortens the life expectancy of the MWFs and lowers the quality of the metal machining. However, this can be exploited in future MWF formulation to aid end-of-life biotreatment. Conversely, compound D1 (a biocide, EBC-1) was the key determinant in terms of restricting rates of degradation. Therefore, to improve the quality of the synthetic MWFs in term of extending the life of the fluid, it is suggested that compound A12 is replaced by alternative compound. In collaboration with BP chemists, it was proposed to remove compound B3 (an acid, Corfree M1) since the analysis here classified it to be “soft” in terms of being conducive to stimulating biodegradation. B3 (an acid, Corfree M1) was a constituent of the formulation added as a corrosion inhibitor, which has the same function as other acids in the formulation such as B10 (Neodecanoic acid) and B30 (an acid, Cobratec TT50S). However, because B10 was “harder” in terms of being non-conductive to biodegradation (as can be

seen in Figure 3.14 and Figure 3.20), so B3 (an acid, Corfree M1) was removed and replaced by B10. By such a substitution the quality of MWFs should be maintained in terms of longer performance and it is expected to be more resistant to bio-deterioration. The research was continued in section 3.3.3, amine substitution, to observed best alternative option in the Syntilo 9913 formulation in term of "biohardening".

3.3.3 Amine substitution (Case study)

In this work, other types of amines were studied to improve the biohardness of synthetic MWF, Syntilo 9913. From the combinatorial studies (see section 3.3.2), compound A12 (Triethanolamine) was found to be a predominantly "softening" agent, that is to say the key component, which contributed to stimulating premature biodeterioration. To overcome this potential for premature deterioration, therefore, other amines were substituted with the aim to improve the "biohardness" of the existing synthetic MWFs, Syntilo 9913. In addition, the effect of compound B3 was also observed in this experiment to be a predominant softening agent (Figure 3.20). The removal of biocide compound, D1, was also tested to observe the potential of replacing its biocidal impact by employing an amine within the formulation which acted as an antibacterial agent, so enabling the MWF to be a biocide-free product.

From the results, amines were found to behave differently in the presence and absence of D1 (a biocide, EBC-1) and B3 (an acid, Corfree M1). This is useful information for future formulation chemists since it pin-points which amines stimulate of inhibit rates or biodeterioration. To aid this, the amines are categorised in Table 3.11.

Table 3.11: The effects of the presence compound B3 and D1 on the relative biodegradability of the amine formulation are shown below:

Group A. and B. were the most biologically resistant in the absence of D1 and B3,

C. was the most biologically resistant in the presence of D1, and

D. was the most biologically resistant in when both D1 and B3 were presence.

(More details are explained below)

A	**B**	**C**	**D**
Octylamine (A6) + Corrguard LSA8000 (A10)	Octylamine (A6) + TEA (A12)	Synergex T Plus (A14)	Corrguard EXT (A7) + Corrguard LSA8000 (A10)
Corrguard LSA8000 (A10)	TEA (A12)	MDEA (A11)	
M.I.P.A. (A4)	Corrguard EXT (A7) + TEA (A12)		
5-Amino-1-Pentanol (A20)	AB95 (A8)		
Product 7910 (A22)	MEA (A1)		
Diglycolamin (A3)			
Corrguard LSA8000 (A10) + Synergex T Plus (A14)			

From the analysis, the amines used in the formulation can be categorised into 4 groups; A, B, C, and D.

Group A – this group of amines performed best in terms of resisting bio-deterioration in the absence of D1 and B3, and most biodegradable when D1 (biocide) was present in the formulation.

Group B – this group of amines performed best in terms of resisting bio-deterioration in the absence of both D1 and B3, and most biodegradable when both D1 and B3 was present in the formulation

Group C – this group of amines performed best in terms of biological resistant in the presence of D1 (biocide), but was most susceptible biodegradable when both D1 and B3 present together in the formulation

Group D – this combination of amines demonstrated most biologically resistant when B3 and D1 were present in the formulation, and most biodegradable when only D1 was added to the formulation on its own

This confirms that in some instances the amine component of the MWF made MWF became more biologically resistant in the presence of B3 and D1 and another instances it made it more vulnerable.

From this study and analysis, it suggested that combination A6.10 (compounds A6, A10, B9, B10, B30, B31, and B32; see Appendix III) was the best combination in term of its resistance to biodegradation and could be proposed as a new synthetic MWF formulation. This combination consisted of 7 chemical constituents instead of 8. The combination contains 2-amines mixture, acid, and a polymer and most importantly it is a biocide free formula but maintained a high level of antimicrobial activity. This is an important result since it suggests that it is possible to formulate a new MWF that avoids the requirement for a biocide, which has a lot of implication to the manufacturers. In terms of formulating a new synthetic MWFs this combination has additional advantage of containing fewer compounds which would mean lower material and administration costs for production (U.S. Environmental Protection Agency, 2012). Producing a biocide free MWFs is a key long

term aim for manufacturers', as the use of biocide is problematic in terms of health and disposal, plus they are expensive to make and manage. For instance, in the process of registration of new products with biocide it can cost $10 Million and take over 5 years (Reisch, 2012). Furthermore, some biocides can no longer be used because of their toxicity or long term persistence (The Biocide Directive 98/8/EC, 16 Feb 1998), which can be the limitation to the manufacturers that rely on the biocidal MWFs.

Brutto (2011) has reported that amines that have a carbon composition ranges from 8 to 12 carbons with inclusion on hydroxyl group are best for suppressing biodeterioration and corrosion. Amines are easier to manage at the end-of-life compared to biocides as amines can be broken down both aerobically and anaerobically (Frings *et al.,* 1994; Williams and Callely, 1982). The combined anaerobic and aerobic degradation pathways typically include NH_4 and NO_3 as intermediates and N_2 as the final product of denitrification. The key feature of biological cell membranes is their amphiphilic characteristic, so having hydrophilic group helps partitioning water to lipid layers and can cross through the membrane and into the cell (Golec *et al.,* 1989). This agreed with the finding in this study, A6 (Octylamine) was as an effective antimicrobial agent as it had a linear structure and is hydrophobic compared to A12 (Triethanolamine) (Golec *et al*., 1989). However, according to the market price, the cost of A6 is not economically sustainable. Moreover, compound A6 has 8 carbons and no hydroxyl groups, hence it could be employed as "hardening" agent in the newly formulated MWFs, whilst amine A12 has 3 hydroxyl functional groups, which could be the factor in term of stimulating bacterial degradation (Brutto, 2011) as observed in this study.

Therefore, the original data from combinatorial screening was used and it was found that combination 5.22, which composed of A12, B9, B10, B30, and B32 (see Figure 3.21: Top 50 most recalcitrant combinations in terms of resistance to biodeterioration) was found to be the most suitable as the mixture that was most resistant to bacterial growth. It contained chemicals that will give the same function as the original formula, but importantly the biocide was omitted. It contained fewer compounds, making it more cost effective than the original formula. From the collaboration with BP-Castrol, it was confirmed that compound B3 (acid, Corfree M1) can be replaced with B10 (acid, neodecanoic acid); compound B31 (polymer, Pluronic 17R20) is limited availability in the market and can be replaced with B32 (polymer, Pluronic 17R40); and compound D1 (EBC-1 biocide) is toxic and biocide free formulation is much preferred. Hence, this formulation is planned ahead in term of future change in raw material availability and it is named Syntilo 1601 as a reformulated synthetic MWF, which has been taken forward to further degradation study in Chapter 4.

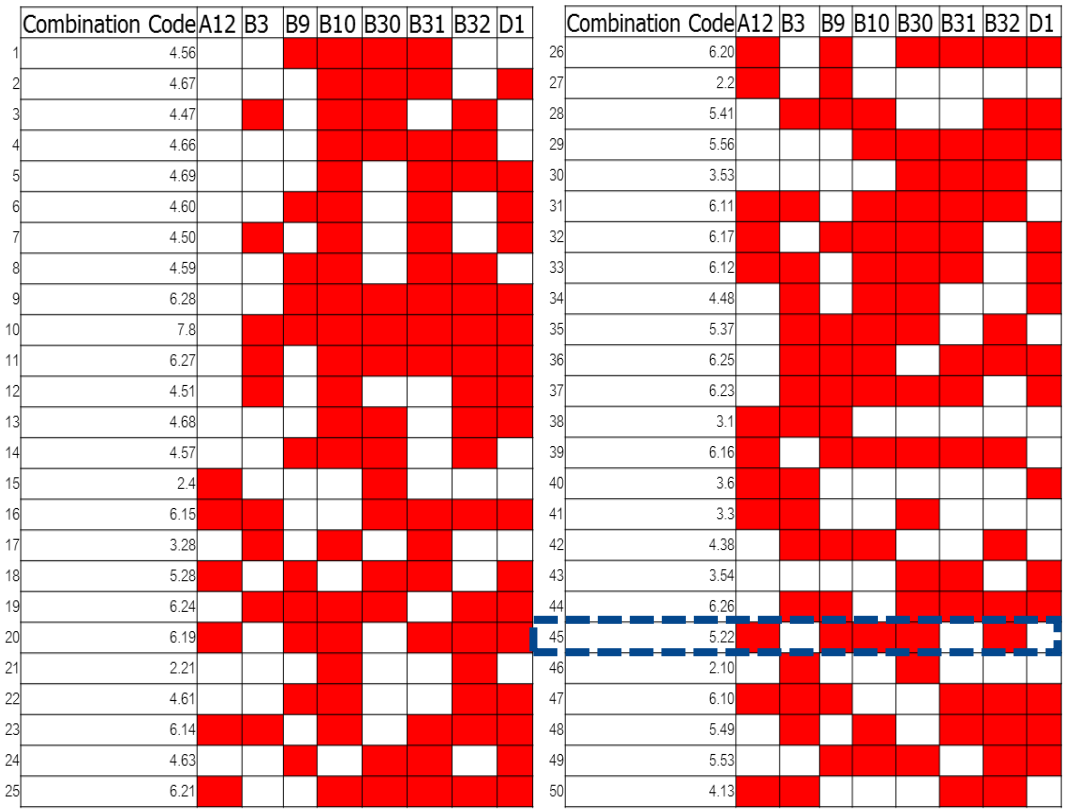


Figure 3.21: Top 50 most recalcitrant (hard) combinations in terms of resistance to biodeterioration

3.4 Conclusion

Each of the 80 generic MWF chemical constituents had their own distinctive behaviour in terms of toxicity towards microbial cells and their biodegradability. The compounds were categorised and summarised in Table 3.10 as lethally toxic, inhibitory, recalcitrant, benign, and toxic in terms of the activity of biosensor assays employed in this study. The data for the characteristics of the individual MWF chemical components enabled predictions to be made in terms of their chemical fate as individual component and when present in mixtures of increasing complexity. Subsequent analysis of component combinations could then be compared and eventually predetermined fate and employed allowing predictive models to be developed. Those data then can be plugged into the model and predict toxicity and degradability. Such an *in silico* approach would reduce MWF development costs significantly. Subsequently, the effect of chemical interaction toward toxicity and degradability were further studied in combinatorial study. It will also enable sustainable end-of-life to be tailored made according to the predetermined characteristics of the MWF formulation.

A typical synthetic MWF such as the BP-Castrol Syntilo 9913, which was investigated here, consists of eight of the components generated from a formulation palate of at least 80 generic chemical options. Due to chemical interaction, the predicted toxicity using single screening data altered. Therefore, Syntilo 9913 was studied by applying a reverse engineering approach starting with 2 constituents and then increasing in complexity, resulting in the testing of 255 possible combinations of 8 components (Equation 2 and Table 3.5). From these studies, it was found that compounds A12 (Triethanolamine) and B3 (an acid, Corfree M1) were pivotal constituents, with regards to stimulating biodeterioration, and so were referred to here as “softening agents”, whilst compound D1

(EBC-1 biocide) was the predominant constituent in terms of inhibiting biodeterioration and referred to as “hardening agent” (Figure 3.20).

Furthermore, from the combinatorial studies (section 3.3.2), it is clear that the predominantly “softening agent” for the product Syntilo 9913 was identified to be A12 (Triethanolamine), the presence of which clearly encourages premature biodeterioration (Figure 3.19 and Figure 3.20). This shortens the life expectancy of the MWFs and lowers the quality of the metal machining, which is not ideal when it is in operations. If the objective of the manufacturer were to extend the working life of the MWF, this constituent should be replaced with a mere recalcitrant compound. This hypothesis has been tested by removing A12 and substituting with other amine options (Table 3.6), which was less susceptive to biodegradation. Ten amine options were carefully selected in collaboration with BP-Castrol by considering the solubility and other key properties of the product. As shown in the Table 3.6, amine A6, A7, and A14 have to be solubilised with other amines at low concentration at a ratio to accommodate the solubility of the final product. Compound B3 (an acid, Corfree M1) and D1 (a biocide, EBC-1) were also substituted. This was because B3 (an acid, Corfree M1) was found to be ‘softening key’, which is not an essential constituent in the formulation as its function, corrosion inhibitor, which can be replaced by other acid such as B10 (neodecanoic acid). D1 was substituted to test the biocide-free formulation as the biocide is very toxic and it is both time and cost intensive to register a biocidal product. Also, a key future issue is the need to comply with the Biocide Directive (EU 98/8/EC), which seeks ultimately to eliminate biocide use. As a result, combination A6.10 (Octylamine, Corrguard LSA8000, Isononanoic acid, Neodecanoic acid, Cobratec TT50S, Pluronic 17R20, and Pluronic 17R40), which did not contain B3 (an acid, Corfree M1) and D1 (a biocide, EBC-1), was found to be the ‘hardest’ in term of resistance to biodeterioration. A6 (Octylamine) and A10 (Corrguard LSA8000)

were found to be the best amine options to replace A12 in terms of "hardening" the MWF formulation and maintain the same role as corrosion inhibitor, emulsifier, and surfactant. However, according to the market price, the cost of A6 is not economically sustainable.

Further data analysis revealed that combination 5.22, which composed of A12 (TEA), B9 (Isononanoic aicd), B10 (Neodecanoic acid), B30 (Corbratec TT50S), and B32 (Pluronic 17R40) from the combinatorial data, was the best candidate to take for further biodegradation study in Chapter 4. This was supported with four reasons;

1. The cost of compounds were economically sustainable for the manufacturer;

2. It is a biocide free formulation and resistant to biodeterioration compared to the original formulation (growth < 0);

3. This formulation has only 5 components, which means it is greener according to green engineering principles 1 (Inherent Rather Than Circumstantial), 2 (Prevention Instead of Treatment), 7 (Durability Rather Than Immortality), 9 (Minimise Material Diversity), and 11 (Design for Commercial "Afterlife") (ACS, 2014); and

4. Compound B31 (Pluronic 17R20) is limited in the market and its exclusion from the formulation, reduce the challenge of trying to source a component that is in the limited supply.

The information gained from this chapter confirmed one of the key factors determining that the susceptibility of the Castrol product Syntilo 9913 to premature biodeterioration was due to the presence of Triethanolamine (A12), which was found to stimulate microbial

growth. Studies in which this was reformulated confirmed that its susceptibility to premature biodeterioration could be reduced if TEA is replaced. However, such reformulation would increase the product “hardness” in term of biodegradation and inhibit or certainly slow down the effluent treatment for end-of-life treatment using bio-treatment. This contradiction has been investigated in more detail in Chapter 4, biodegradation studies. As a part of future work, cooperation with the chemists will be required to look at the structure chemistry relationship and integrate the chemistry with biological in order create more predictive model.

CHAPTER FOUR:

DISASSEMBLY OF A MWF PRODUCT TO IDENTIFY "BOTTLENECK" COMPOUNDS (SYNTILO 1601)

CHAPTER FOUR :

DISASSEMBLY OF A MWF PRODUCT TO IDENTIFY BOTTLENECK COMPOUNDS (SYNTILO 1601)

4.1 Introduction

MWF constituent compounds as described in section 2.1.2. (Chapter 2) are metabolised via different degradation pathways. For example, amines break down to ammonium and fatty acids and are further catabolised through tricarboxylic acid cycle (TCA) (Figure 2.7) (Knapp *et al.,* 1996) and polymers are degraded to lower molecular weight molecules (de Lucas *et al.,* 2008). Thus, MWFs that have been exposed to bacterial consortia contain a complex mixture of components and their breakdown products from biodegradation processes. Bacterial consortia have been assembled and employed to degrade MWF components and mixtures test compounds. In this case, one strain may be able to degrade an individual component to some degree, and another can continue to degrade it further to smaller molecules (Mo *et al.,* 1997). For instance, many breakdown products are further metabolised, as is the case for acetate, which is produced from ethanolamine (MEA) degradation, and so are not observed to accumulate. However, some products of metabolism accumulate until they cause feedback inhibition of metabolic processes or other adverse effects on the physiology of exposed cells. These metabolites are often referred to as "bottleneck" compounds because they limit metabolic flux. "Bottleneck" compounds are of particular interest for two reasons. Firstly, they limit the degree of biodegradation of the operationally exhausted MWFs in wastewater treatment plants and secondly, because they may potentially be purified and employed as antimicrobial compounds in potential future products.

Liquid chromatography (LC) was employed as the main tool to identify "bottleneck" compounds in the biodegradation process of three test formulations BR1, BR2, and modified synthetic MWF Syntilo 1601 (see Chapter 3). Sample BR1 and BR2 were simple base formulations, developed specifically by BP Castrol, consisting of acids and amines, which was designed to allow protocol development and identification of specific components and their breakdown products. With this simplified system the rationale was that it should be easier to understand the component mixtures in formulated Syntilo 1601 (Table 4.1). The accumulating "bottleneck" compounds were then analysed by liquid chromatography and identified according their elution times. This information will be applied in Chapter 5 to enabled focused research on their immobilisation and removal of selected components to aid cradle-to-grave design at the end-of-life.

4.1.1 Aims and objectives

Synthetic MWFs are typically difficult to degrade as they lack a readily degradable carbon source (Connolly *et al*., 2006; Rossmore, 1989). Therefore, this work was carried out to gain insight into the biodegradation of Syntilo 1601, the modified formulation resulting from preliminary work (described in Chapter 3) of a BP Castrol product and a key commercial product for our collaborative partners. Individual MWF components and their breakdown components were detected and monitored during the biodegradation process using liquid chromatography (see section 4.2.3.2) by observing the formation and disappearance of peaks. Then, overall degradability was determined employing the reduction of COD levels and the production of ammonium and nitrate employing Hach Lange Kits (see section 4.2.3.1). This work helped to gain insight onto the production of the "bottlenecks", which then focused upon further in Chapter 5.

4.2 Materials and methods

4.2.1 Sample preparation

Pilot samples, BR1 and BR2, and modified MWF (Syntilo 1601) were prepared according to Table 4.1. These combinations were chosen since BR1, and BR2 were previously found from the research in the group to represent the polar extremes in terms of the softest (BR1) and hardest (BR2) combination of one amine and one acid, respectively.

Table 4.1: Composition and concentration of test samples

Test Samples	**Chemical compounds and concentration (the letters indicate compound code for each chemical constituent)**
BR1	Caprylic acid (10%) + Methanolamine (10%) B8 A1
BR2	Neodecanoic acid (10%) + Corrguard LSA 8000 (10%) B10 A10
Syntilo 1601 (combination 5.22)	A12 (15%) + B9 (2.5%) + B10 (5%) + B30 (2%) + B32 (30%)

4.2.2 Biodegradation test

The synthesised MWF samples were diluted to 5% v/v in conical flasks with additional of M9 minimal growth media (11.28g/L), Magnesium Sulphate, MgSO4 (0.56g/L), Glucose (5g/L), Sucrose (5g/L). In addition, 1.2% v/v of resuscitated mid-exponential acclimated bacteria from freeze dry stock (section 3.2.4) was added to each flask as an inoculum. The flasks were kept at 35ºC and 120rpm in shaking incubator (Innova 44, New Brunswick Scientific) for 14 days. To determine the biodegradability of the samples, specimens were

taken every other day using a 5 mL syringe (BD Plastipak), and filtered using a 0.2 µm filter (Thermo Scientific). Biodegradation was tested by measuring COD, ammonium, nitrate, and chemical analysis by liquid chromatography (LC).

4.2.3 Analytical techniques

MWF degradation can be monitored using several techniques such as measuring chemical oxygen demand (COD), total organic carbon (TOC), and chemical breakdown product analysis by employing High Pressure Liquid Chromatography (HPLC), Gas Spectrometer (GC), or Mass Spectrometer (MS). Measurement of COD allows the overall process of microbial oxidation to be observed. COD is the main sewage discharge consent parameter, which is widely applied in Modgen Formula to calculate the trade effluent and so is of primary importance to waste processors (see Equation 1 and section 2.1.4).

4.2.3.1. Chemical oxygen demand, nitrate, and ammonium measurement

COD, nitrate and ammonium were measured using a commercially available colorimetric test according to manufacturers' instruction HACH LANGE LCK014 (COD: 1,000-10,000 mg/L O_2), LCK340 (Nitrate: 5-35 mg/L NO_3-N), and LCK304 (Ammonium: 0.015-2.00 mg/L NH_4-N) cuvette tests (HACH LANGE, n.d.). The values are reported in terms of percentage removal during the biodegradation process.

COD measurement

Chemical Oxygen Demand (COD) is widely used in environmental chemistry as a surrogate tool for measuring water quality (Benitez *et al*., 1999) or precisely, the amount of organic pollutant found in the water is expressed in milligrams per litre, which indicates the mass of oxygen consumed per litre of solution to fully oxidise it. This is done by calculating the amount of oxygen that is required to oxidise the organic content to carbon dioxide using strong oxidizing agent under acidic conditions. The value of COD is inclusive of the oxygen demand for oxidizing ammonia into nitrate (i.e. nitrification). The process of ammonia being converted into nitrate is referred to as nitrification.However, for '"bottleneck"' that is recalcitrant compound identification, COD measurement does not provide information on the fate of the individual MWF components as they degrade. Liquid chromatography - Mass Spectrometry is, therefore, ideal to employ in addition to analyse individual chemical breakdown products during degradation process. However, due to the limitation of availability of instruments, LC was used in this research (The details of LC method can be found in section 4.2.3.2). In addition, LC has been used extensively as an efficient analytical tool for chemical analysis of different classes of substance, such as in medical science and food science (Ito and Fujita, 1985; Khachik *et al*., 1986).

4.2.3.2. Chemical analysis by liquid chromatography (LC)

Liquid chromatography (1120 Compact LC, Agilent) was employed to detect chemical disappearance and formation in order to create a biodegradation profile and identify "bottleneck" compounds for BR1, BR2, and Syntilo 1601 in 14 days. One millilitre of filtered prepared sample was injected into the system (Figure 4.1) via the auto-sampler. Distilled water and acetonitrile were used as mobile phase and Purospher STAR RP-18

endcapped column (Merck) was used as a stationary phase in this experiment. The analysis carried out for 30 minutes in gradient, where the concentration of acetonitrile gradually increased.

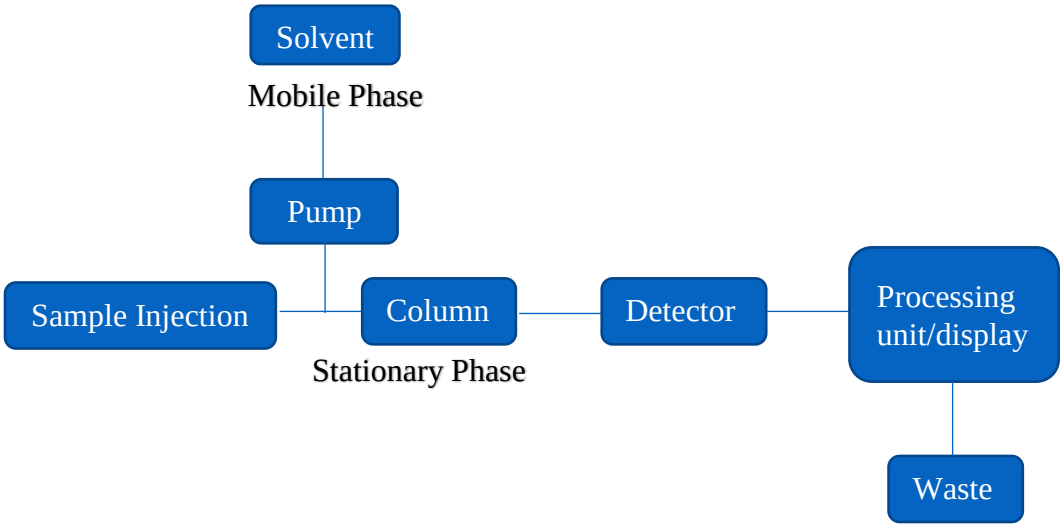


Figure 4.1: A Flow Scheme of Liquid Chromatography Operating System

4.3 Results and discussions

4.3.1 Degradation of binary mixtures (BR1 & BR2)

From the chemical analysis using LC, it was found that the biodegradation process of MWF is a dynamic one, an example is shown in Figure 4.2. Figure 4.2 shows the peaks that appeared and subsequently disappeared due to catabolic and anabolic activities. For instance, at retention time 3.62 minutes, there was no peak on Day 0 but one did appear later on Day 12, when a very tall peak was formed, and peak height was lowered on Day 14; this is an example of the degradable by-product of the metabolic activity. This analytical technique has been used by many researchers (Keppler and Humpf, 2005; Anderson *et al.*, 2009; Dong *et al.*, 2015; Nakajima-Kambe, *et al.*, 1995). For example, Nakajima-Kambe *et al.* (1995) employed HPLC and detected one large and one small peak

as metabolic breakdown product of polyurethane, which corresponded to be diethylene glycol and trimethylolpropane, respectively.

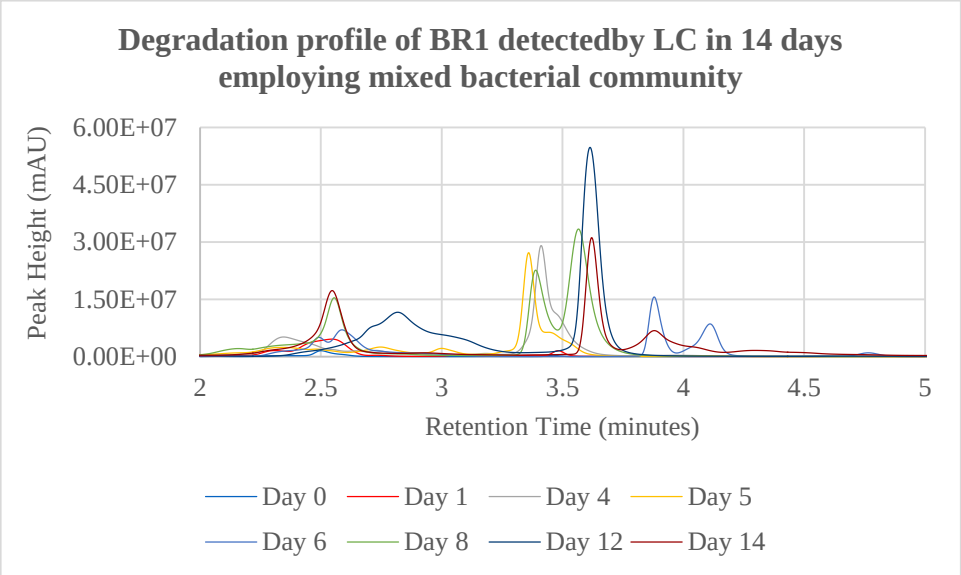


Figure 4.2: Example of closed up raw data (at retention time of 2-5 minutes) of dynamic biodegradation process of BR1 detected by LC in 14 days employing mixed bacterial community.

Figure 4.3 represents the dynamic combinatorial chemistry interaction of sample BR1 (Caprylic acid and MEA) and BR2 (Neodecanoic acid and Corrguard LSA8000) after 7 days of biodegradation. A greater number of peaks were detected in chromatograms after exposure to bacteria (Day 7) when compared to those at the starting point of the experiment (Day 0). Those peaks which were easy to biodegrade disappeared and recalcitrant or toxic components accumulated over time.

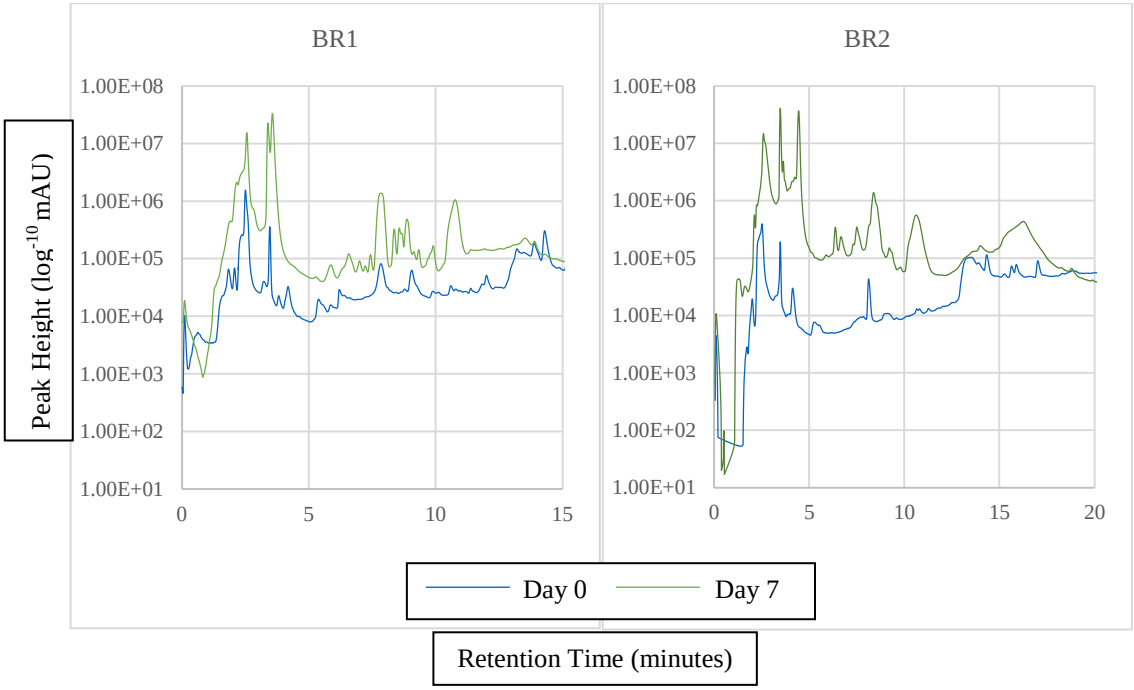
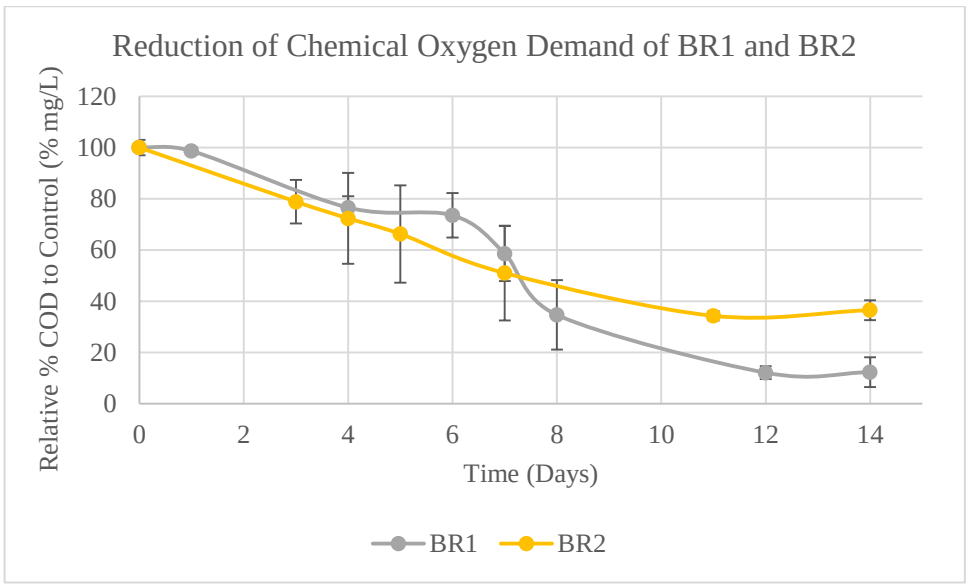
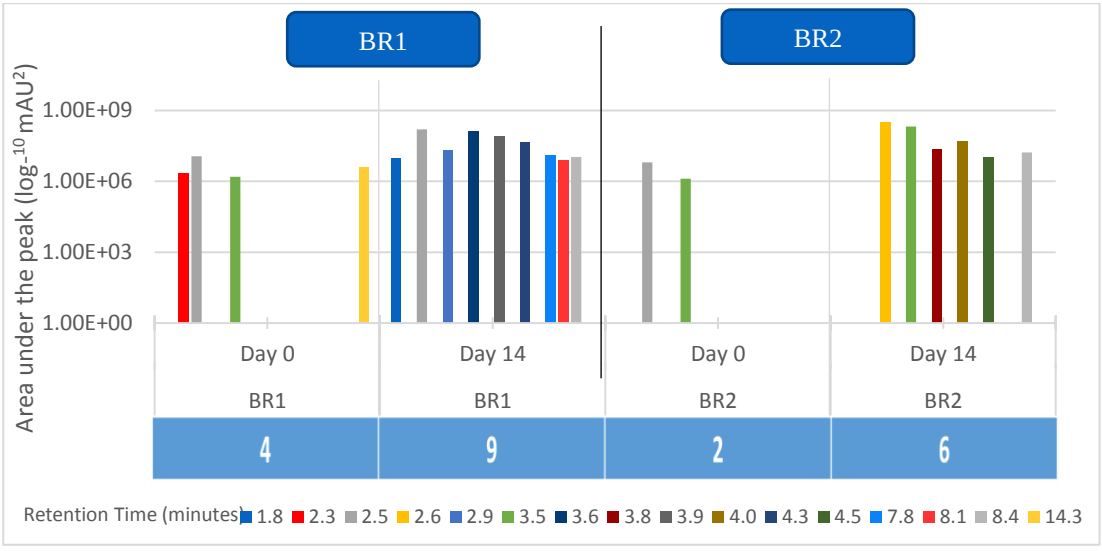


Figure 4.3: The chromatograms show the formation and disappearance of biodegradation by-products.

The area under the peaks detected in the chromatogram was determined and plotted as a bar chart (Figure 4.4 (b)). As seen in Figure 4.4 (a), BR1 showed higher COD removal after 14 days compared to BR2 by approximately 20%, as expected, due to a 'softer' composition. 'Harder' compositions enable minimal COD reduction (Laopaiboon *et al.*, 2002) A prediction was made from the single screen data (see Table 3.10 and Figure 3.12), it was found that both compound in BR1 (A1 - MEA and B8 – Caprylic acid) stimulated high bacterial growth, whereas compounds in BR2 (A10 – Corrguard LSA8000 and B10 - Neodecanoic acid) suppressed bacterial growth. However, from the number of peaks detected in the LC spectrum, it appears that a larger number of products were formed with the 'softer' formula. The number of peaks from degrading BR1 and BR2 increased by 5 and 4, respectively from day 0 to day 14. This may have been due to the BR1 being a softer product and biodegradation proceeds on various pathways, resulting in the formation of more products in comparison to BR2.



(a)



(b)

Figure 4.4: COD removal trends for BR1 and BR2 (a), LC chemical analysis of BR1 and BR2 after 14 days (b)

Information that was obtained from the LC analysis for the biodegradability of each substance allowed degradable and non-degradable compounds to be distinguished. Figure 4.5 is a close-up biodegradation profile of sample BR1 during the first 14 days under aerobic conditions. Four retention times, 2.5, 2.6, 3.5, and 3.7 minutes, were selected as

examples of biodegradable and recalcitrant compounds, which could be either raw materials or by-products. The following definitions were used:

- Biodegradable raw material: peak appears at time 0 and subsequently disappears
- Recalcitrant raw material: peak appears at time 0 and remains through the experiment
- Biodegradable by-product: peak appears after time 0 and disappear
- Recalcitrant by-product: peaks appears after time 0 and remains through the experiment

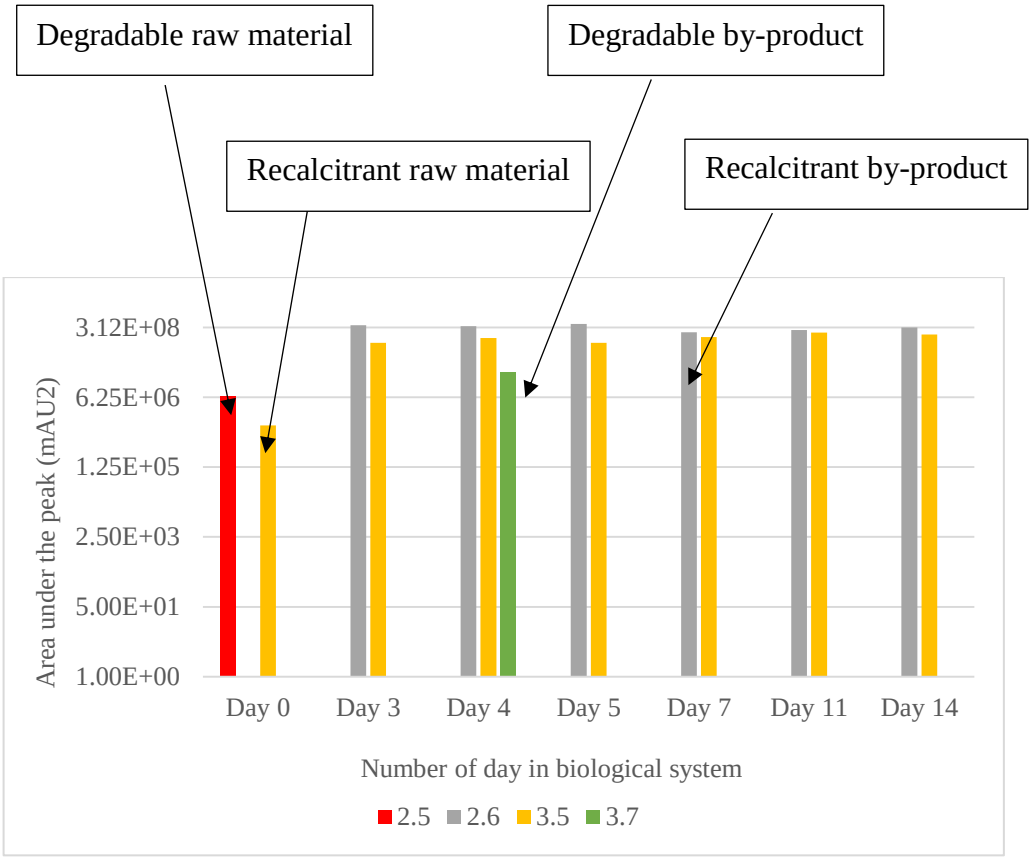


Figure 4.5: Closed up shot of degradation profile of BR2 to illustrate the terms: biodegradable and recalcitrant materials.

Figure 4.4 and Figure 4.5, show the concept of biodegradation of hard (BR2) and soft (BR1) binary mixtures employing mixed bacterial community taken from a long established lab-based bioreactor used for treating waste MWF. This work provides insight into the dynamic the catabolic and anabolic activities during the biodegradation of soft and hard MWFs. This was then, applied in the more complex combination, Syntilo 1601. As described in Table 4.1, Syntilo 1601 is composed of five chemical components; TEA, Neodecanoic acid, Decanonanoic acid, Cobratec TT50S, and Pluronic 17R40. In general more complex formulations are much more difficult to degrade presumably because of the greater number of "bottleneck" compounds or by-products produced.

4.3.2 Degradation of synthetic MWF, Syntilo 1601

As with the binary mixtures test, the degradability of Syntilo 1601 was studied for 14 days and was found that the breakdown process was dynamic. It should be noted that all peaks may not have been detected as the result of wide range of chemical type, some of the compounds were large, insoluble, and but also consisted of small constituents. Hence, a mid-range column was used in this experiment as per specialist's recommendation.

With five raw materials, there were five peaks were detected at time 0. Many by-products were produced from biodegradation of raw materials as shown in Figure 4.7 and Figure 4.8. This technique has also been used by Anderson *et al*. (2009), where it was identified that peaks could be assigned to compounds based on their size and retention time, with smaller compounds such as TEA creating peaks at lower retention times, and larger compounds arising sequentially in order of carbon chain length, for example the peak for hexadecanoic acid (6 carbons) arose prior to octadecanoic acid (8 carbons). It is to be noted that some

intermediates might be undetectable as they were either, not produced in the cell-free system or, they were metabolised too rapidly for possible detection under the experimental conditions employed (Sherburn and Large, 1999). In this study the five compounds were of different size, hence it is likely that the order of peaks versus retention time corresponded to the compound size, with the first peak identifying TEA and the final peak identifying the pluronic 17R40 polymer. Five peaks were detected, however the single compound analysis for each individual constituent identified no peaks. This suggested that the 5 peaks may not have been a result of the initial components, and instead were the result of by-products of degradation.

The possible breakdown products from amine degradation alone could be intermediates of lower ethamolamines such as DEA and MEA, glycolaldehyde, acetaldehyde, and ammonia as Knapp *et al*. (1996) and Sherburn and Large (1999) identified (See Figure 4.6). Furthermore, they reported that Aldehyde was found to degrade 60-100% over time. This suggests that one of the degradable intermediate by-product peaks is Aldehyde in Figure 4.7 and Figure 4.8. Ammonia was continuously detected in the system (Sherburn and Large, 1999), suggesting that it was continually produced or was accumulating similar to the finding in this study (see Figure 4.9c). Three raw materials, at retention times 2.3, 3.6, and 10.3, were found to be degradable as the peaks disappeared over time. However, two of these raw materials are found to be recalcitrant, this could also be due to the synergistic interactions of compounds in combination as shown in Combinatorial Studies (section 3.3.2) Note that chemical may exhibit synergistic or antagonistic behaviour when they interact. On the other hand, this could suggest that metabolic by-products are more or equally toxic to parent compounds (Dong *et al*., 2015; Lin *et al*., 2008). For example, Lin *et al.* (2008) reported that 4-[(6-chloro-2-benzoxazolyl)oxyl] phenol (CBOP), which is a product in the degradation process of herbicide Fenoxaprop-ethyl was found to be more

toxic than the parent compound. For this work, those peaks that remain on day 14 are recorded as possible recalcitrant "bottleneck" compounds, which stop the degradation process. Removal and exploitation of these compounds offers potential for controlling microbial activity so that bacteria are supressed in the MWF during use but can be removed to facilitate bioprocessing. This is the aim of the hybrid technologies applied in Chapter 5 of this work.

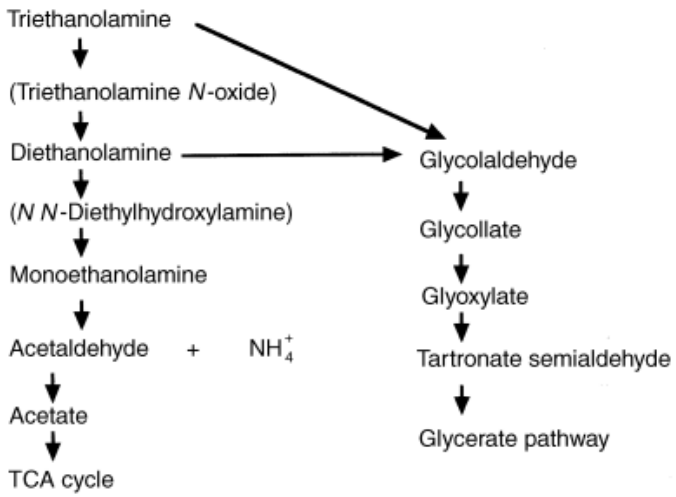


Figure 4.6: Metabolic pathway of alkanoamine, Triethanolamine (TEA) by aerobic and facultative anaerobic bacteria (Sherburn and Large, 1999)

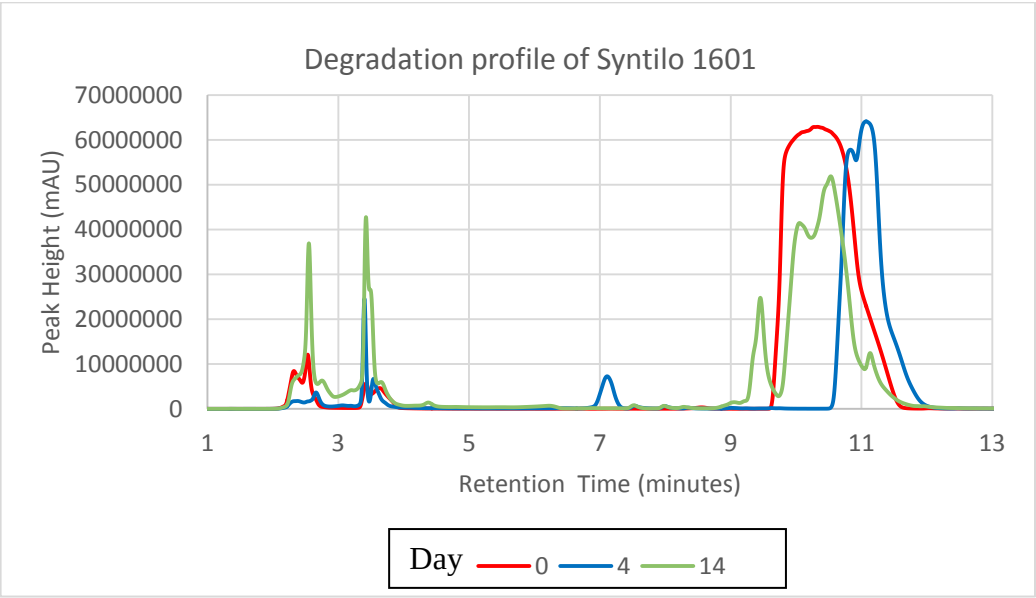


Figure 4.7: Peak detected from LC from degrading Syntilo 1601

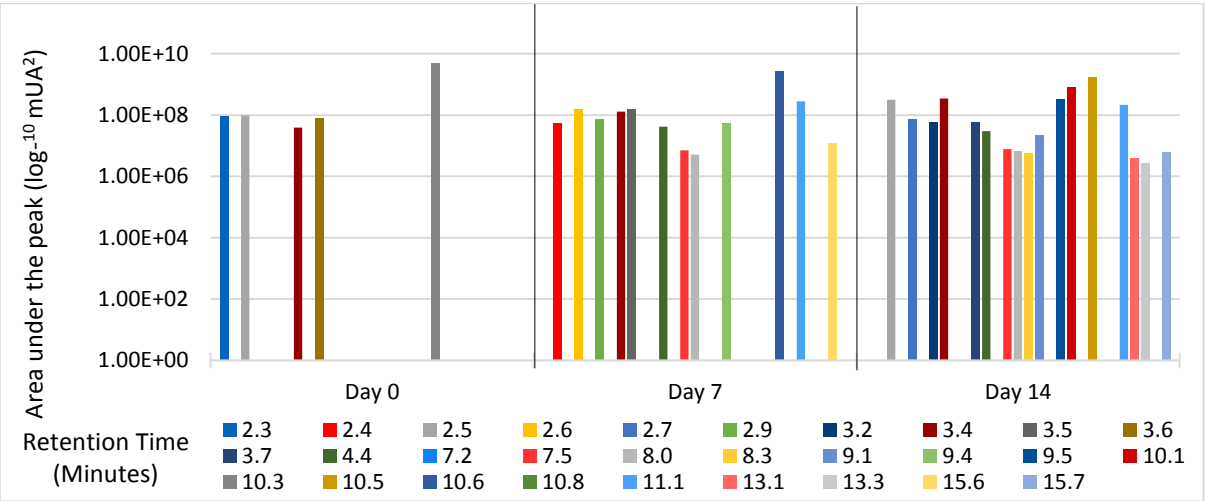


Figure 4.8: Degradation of modified Syntilo (Syntilo 1601) by an acclimated bacterial consortium for 14 days detected by liquid chromatography.

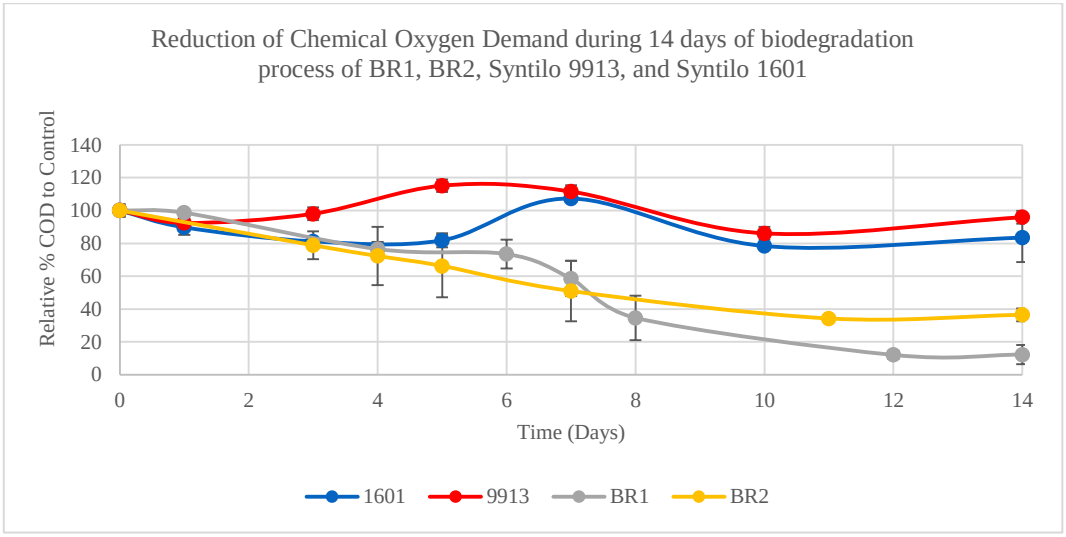
The dominant degradability measure is the COD, as measured and presented in Figure 4.9(a). It was found that often the more complex the mixture is, the harder to degrade. Although BR2 is harder in term of microbial deterioration compared to BR1, but as it only contains 2 compounds in the mixture, it is much easier to degrade compared to the Syntilo 1601 (36.51% and 12.27% COD reduction, respectively). Moreover, although from the combinatorial studies it was shown that modified MWF was harder in terms of biological resistance in the 24 hours growth test in 96 vials format but in terms of biodegradation system it is slightly less biologically resistant than Syntilo 9913 as the COD reduced 16.53% and 4.10%, respectively. This is consistent with previous work that found synthetic MWF to be difficult to biodegrade (BP-Castrol Limited, 2012).

Figure 4.9(b), shows a nitrate reduction of approximately 80% in all samples, which may be due to the consumption of the nitrogen source by nitrifying bacteria in the

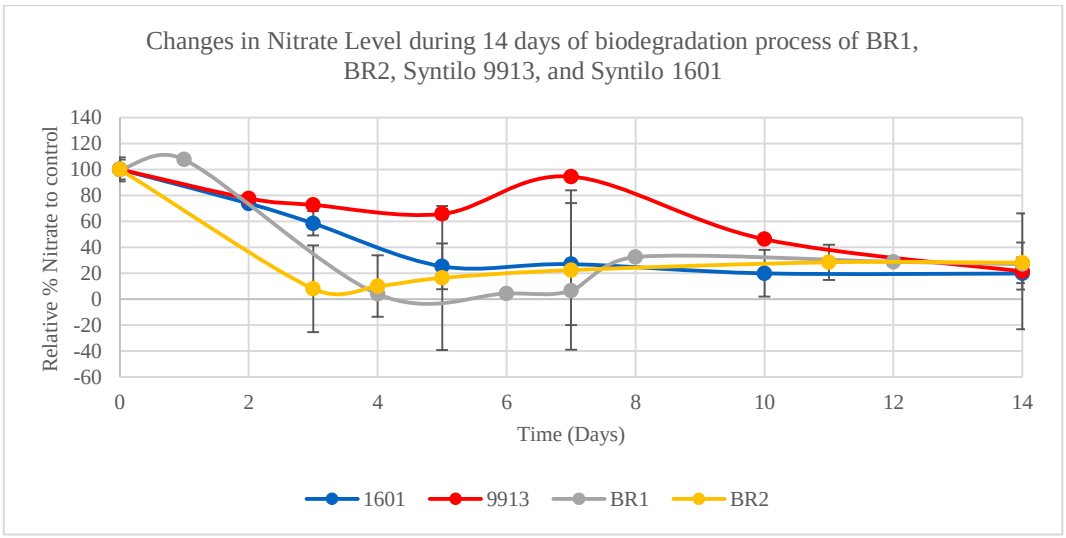
denitrification process as has been observed previously (Sun *et al*., 2009; Eriksson *et al*., 2002; Kim *et al.*, 2001; Alvarez and Vogel, 1994; Yuan *et al*., 2000)

Figure 4.9(c) shows an increase in level of ammonium, which may be an indicator of consumed amine (Kim *et al*., 2001; Knapp *et al*., 1996). Samples BR1 and BR2 composed of 10% of amine in the combination, which is a lower percentage compared with Syntilo 1601. This may be the reason that the level of ammonium is lower in these two formulas compared to Syntilo 1601 and Syntilo 9913. However, the level of ammonium for Syntilo 9913 has shot up from Day 7 to Day 14 continuously, this seems that the production of ammonium could be from the addition of other chemical compounds present in the formula of the formulation of by-products.

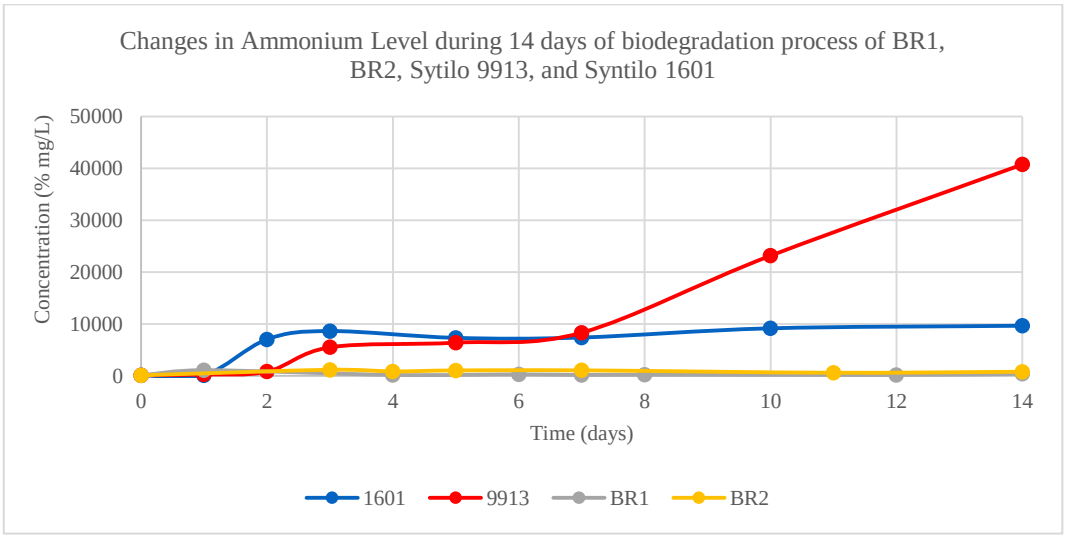
In addition, although from single compound screen, A12 (TEA) was found to be relatively hard compared to other chemical compounds, there was slight growth when exposed to the mixed bacterial community. However, Campo *et al.* (2011) reported that TEA was degradable up to 98%, this disagreed with this study. A possible explanation could be that Campo *et al*. (2011)'s experiment was in saline conditions with bacteria that grow in different type of industrial waste bioreactor. Kim *et al. (*1994) reported that primarily alkanolamines can be degraded through aerobic treatment. Tertiary amine does not seem to degrade easily biologically. This was in agreement with the results in this study (Figure 4.4a). Connolly *et al*. (2006) reported that unknown amines demonstrated to be difficult to degrade and only achieved by reduction of 5% in the ethanolamine content in the reactor.



(a)



(b)



(c)

Figure 4.9: Changes in the level of COD (a), Nitrate (b), Ammonium (c). Tests were done in triplicates. Error bars represent the standard deviation of the mean *(n=3)*

4.4 Conclusions

In the work in this chapter, during the biodegradation of MWF, chemical compounds form and disappear during catabolism and anabolism, respectively. As expected, the "soft" formulation (BR1) metabolised quicker than the "hard" one (BR2) as measured by COD. However, as both samples, BR1 and BR2, were simple binary mixture unlike Syntilo 1601, they generally degraded easily. In the more complex systems such as Syntilo 1601, the metabolic activity was observed to be much more dynamic compared to binary mixtures.

The results from ammonium measurement confirmed that there was metabolic activity resulting in the degradation of amines from MEA in BR1, Corrguard LSA8000 in BR2, and TEA in Syntilo 1601 and Syntilo 9913. Similarly, ammonia production was observed by Knapp *et al.* (1996), and Sherburn and Large (1999). Knapp *et al*. (1996) reported that tertiary amines are broken down into diethanolamine (DEA), and then degraded into ethanolamine (MEA). There is a debate on the rate of degradation of TEA, where Knapp *et al*. (1996) observed that anaerobic undergo at a faster rate, where Bio-Wise (2010) argued that aerobic condition is more efficient. For work in this study, aerobic conditions were maintained. The aerobic process is catalysed by mono-oxygenases and so it is assumed here that the aerobic pathway was followed LC peaks represent aerobic degradation product from the pathway in Figure 2.6 and Figure 2.7). Further degradation of MEA produces acetaldehyde which is oxidised into acetic acid and then enter tricaboxylic acid cycle. Denitrification of nitrate was also observed in this study (see Figure 4.9b). This could have been due to the presence of denitrifying bacteria or the consumption of carbon source in denitrification process. Knapp *et al.* (1996) has also found the similar results where the reduction of nitrate was observed by 22% in aerobic system.

COD measurements confirmed that the modified Syntilo 1601 was as biologically resistant as the original formulation, Syntilo 9913 another Castrol product, which agreed with the work of Rossmore 1989 that synthetic MWF is difficult to degrade. Moreover, 17 peaks on Day 14, which did not degrade especially peaks at retention time 3.4, 4.4, 7.5, 8.0, and 11.1. These peaks are the targets for the removal with immobilisation technology in Chapter 5. In this work, it was asserted that MWF can be made without biocide still maintain product life (Chapter 4, Figure 4.9a). Chemical interactions that occur in the complex mixture have been shown to make MWF recalcitrant without the need for a biocide. Some non-lethal chemicals could interact and deliver bacteriocidal property without the need for biocide addition (Chazal, 1995; Collier *et al.*, 1990; The Biocide Directive 98/8/EC). Biocide removal is both economically and environmentally sustainable as the design is cradle-to-grave in terms of known solution to end-of-life hybrid treatment.

Work in this Chapter tested the bio-harness of reformulated Syntilo 1601 BP-Castrol product. Results indicated that the reformulated product, which contains no specific antibacterial component, was as bio-hard as the original formulation, which contained EBC-1 biocide. Despite the removal of biocide component, the product is still as bio-hard this is desirable for MWF whilst in operation. However, this is not consistent with the requirement for biological degradation. Therefore, in the next Chapter, immobilisation agents were employed to remove recalcitrant LC peaks identified in section 5.3.2.

CHAPTER FIVE

IMMOBILISING THE TOXIC COMPONENTS IN ORDER TO REDUCE MICROBIAL INHIBITION

CHAPTER FIVE :

IMMOBILISING THE TOXIC COMPONENTS IN ORDER TO REDUCE MICROBIAL INHIBITION

5.1 Introduction

Industrial wastewaters are often resistant to biological degradation because they contain organic contaminants that are toxic and harmful to individual organisms and ecosystems. Consequently, the removal of such problematic organic contaminants from the waste streams has become essential, not least to protect exposed end-of-pipe biotreatment systems (Hameed, 2009; Jiang *et al*., 2006; Huang *et al*., 2010; Pang *et al*., 2011a). Significant progresses in wastewater treatments such as filtration, advanced oxidation process, photocatalytic, oxidation, adsorption, and bioremediation have been made to address the problem of water pollution (Huang *et al*., 2006; Zelmanov and Semiat, 2008; Long *et al.*, 2011; Pang *et al*., 2011a, 2011b, Jagadevan *et al*., 2011). However, each method has different drawbacks such as processing efficiency, operational methods, energy and resource requirements, and cost effectiveness.

Synthetically designed products such as MWFs are designed to be toxic, *i.e.* inimical to microbial life, and therefore biologically resistant, to prolong tool life in operation as described in Chapter 3, thus biological treatment is an ambition in terms of sustainability but difficult to achieve if the waste stream is toxic and/or recalcitrant ineffective as shown in Chapter 4. Therefore, there has been much research focused on hybrid technologies aimed at combining physical, chemical, and biological processing to deliver the best

solution in terms of environmental and economic sustainability. For instance, hybrid aerobic-anaerobic treatment has been successfully used for degrading organics and to remove nitrogen in wastewaters to meet the standards required for discharging waste effluent into receiving waters and the environment (Im *et al*., 2001; Abeling and Seyfried, 1992; Walker, 2000; The Water Services Regulation Authority, 2005). Fenton's reaction has been combined with biodegradation to improve the degradation performance up to 70% reduction of COD of reactors processing MWFs waste (Jagadevan *et al*., 2011). In this study, immobilisation agents were combined with the biodegradation processes with the aim of removing recalcitrant/"bottleneck" chemical components and aid further biodegradation of the whole waste.

5.1.1 Toxicity immobilisation agent

There has been an extensive use of immobilisation agents in various applications. For example, activated carbon, clay, zeolites, siliceous material, agricultural wastes, industrial waste products, biosorbents, and others such as starch, cyclodextrin, and cotton (Crini, 2006). One of the common environmental applications is for large scale environmental disaster events such as in the Fukushima Daiichi nuclear disaster in 2011 (Sato *et al*., 2011), and the oil spill incident in the Gulf of Mexico in 2010 (Zhao *et al*., 2010). High levels of radioactive caesium and other undesired materials in the seawater as a result of the disaster was adsorbed by strategically placing zeolite sandbags into the affected areas (World Nuclear Association, 2015; Dave and Ghaly, 2011; Itoh *et al*., 2014; Shimura *et al*., 2012; Mercola, 2012; Misaelides, 2011). In addition, organo-clay is often used as an adsorbent to remove oily waste/organic contaminants (Carmody *et al*., 2007; Srinivasan and Fogler, 1990). For example, Srinivasan and Fogler (1990) employed inorgano-organo-

clays to remove pollutants such as benzo(a)pyrene and chlorophenols from industrial wastewaters.

One of the most popular methods for the removal of pollutants from wastewater is liquid-phase adsorption (Crini, 2006). Adsorption is a well-known equilibrium separation process whereby an adsorbent binds molecules by physical attractive forces, ion-exchange, and chemical bonding (Demirbas, 2008; Hashem, 2007; Crini, 2006; Dabrowski, 2001). This method has been widely employed to remove chemical pollutants from wastewater (Crini, 2006). The major advantages of this process are the potential to use low-cost adsorbents, that are effective on a wide range of compounds and metals, flexibility, simple, easily regenerated substrates, available in large quantities, and that they are resistant to toxic components, making them an ideal complement to low energy biological treatments (Xu *et al*., 2012; Zeng *et al*., 2007; Ahmad *et al*., 2009; Rafatullah *et al*., 2010; Demirbas, 2008; Hashem, 2007; Crini, 2006). For example, activated carbon is known to be very efficient and most widely employed in removal of dyes, phenols, pesticides, and other toxic chemicals but it is not economically feasible in many commercial applications (Hameed, 2009; Tan *et al*., 2007; Hameed *et al*., 2007; Kumar *et al.*, 2007; Hamdaoui and Naffrechoux 2007; Daneshvar *et al.*, 2007; Dwivedi *et al*., 2008; Tsai *et al*., 2008; Crini, 2006). Alternatively, nanomaterials (NMs) have also been reported to be an efficient, cost-effective, and environmentally friendly alternative treatment method due to their expansive surface area, which provides a large number of adsorption sites (Friedrich *et al.*, 1998; Dimitrov, 2006; Dastjerdi and Montazer, 2010). High quality treated effluent can be achieved with proper design of the adsorption process (Crini, 2006). In this study, the common immobilisation agents: nano-iron, clay, zeolite, activated carbon, kaffir lime (*Citrus hystrix*) leaves were applied to observe the effectiveness in toxicity removal.

5.1.1.1 Nano-iron

Nanoscale (1 – 100nm) iron is highly reactive, especially in zero-valent form due to its large surface area, magnetic properties that enables it to be exploited for recycling. In addition, it is relatively biocompatible and benign when in the environment. Much research has shown that nanoscale iron particles have a wide range of applications and are effective in the remediation of industrial, ground and wastewater contaminations such as polychlorinated biphynelys (PBCs), heavy metals, inorganic compounds, chlorinated organic compounds, organochlorine pesticides, and other complex compounds (Xu *et al.*, 2012; Fatta-Kassinos *et al*., 2011; Li *et al*., 2011; O'Connor, 1996). It is easily transportable through ground water, allowing for *in situ* treatment. Nano-iron oxides can act as either adsorbent or photocatalyst and convert contaminants into a less toxic form. Several researchers have reported that the oxide form of nano-iron has high efficiency in removal of organic contaminants (Xu *et al*., 2012; Zhang *et al*., 2010; Zhao *et al.*, 2010; Luo *et al.*, 2011), with the benefit of low-cost, strong adsorption capacity, enhanced stability, ability to treat large-volume of wastewater, and low energy separation employing a magnetic field (Hu *et al*., 2005; Carabante *et al*., 2009; Fan *et al*., 2012; Xu *et al*., 2012).

The most common forms of iron oxide in the environment are magnetite (Fe_3O_4), maghemite (γ-Fe_2O_3), and hematite (α-Fe_2O_3) (Cornel and Schwertmann, 1996; Chan and Ellis, 2004; Xu *et al.*, 2012). Nano-iron has also shown potential in combination with biotechnology such as soil remediation and ground water treatment (Huang *et al*., 2003; Roco, 2003; Gupta and Gupta, 2005; Xu *et al*., 2012).

5.1.1.2 Clay

Clay is a natural resource, which is usually prepared from a fine-grained natural rock or soil material with a particle size of <2 μm. There are many types of clay such as allophane, bentonite, montmorrillonite, and kaolin. Many of them have been found to be good adsorbents (Srinivasan and Fogler, 1990; Babel and Kurniawan, 2003; Ghosh and Bhattacharyya, 2002; Almeida *et al.*, 2009; Kurniawan *et al*., 2006). They have been used for a diverse range purposes from clean-up of oil/fuel spills at sea by physical adsorption to anti-diarrheal medications (Adebajo *et al*., 2003).

5.1.1.3 Zeolite

Zeolites are microporous, alum inosilicate minerals, which are often sourced from volcanic rocks (Erdem *et al*., 2004). There are many types of mineral zeolites but the common ones are analcime ($NaAlSi_2O_6.H2O$), chabazite ($(Ca,Na_2,K_2,Mg)Al_2Si_4O_{12}\cdot 6H_2O$), clinoptilolite ($(Na,K,Ca)_{2\text{-}3}Al_3(Al,Si)_2Si_{13}O_{36}\cdot 12H_2O$), heulandite ($(Ca,Na)_{2\text{-}3}Al_3(Al,Si)_2Si_{13}O_{36}\cdot 12H_2O$), natrolite ($Na_2Al_2Si_3O_{10}\cdot 2H_2O$), and stilbite ($NaCa_4(Si_{27}Al_9)O_{72}\cdot 28(H_2O)$). Naturally occurring zeolites contain high levels of impurities, thus, synthetic zeolite is used in commercial applications where uniformity and purity are essential. They are commonly used as commercial adsorbents and catalysts as is it highly porous material for water and wastewater treatment (Widiastuti *et al*., 2008). Thus, zeolites are attractive for commercial applications as they have potential advantages such as the ability to undergo ion-exchange and adsorption, trap contaminants in water and wastewater, low cost, abundant, only require low technology systems, and have regenerative properties (Widiastuti *et al*., 2008).

Zeolites have a very wide range of application such as in laundry detergent, ion-exchange beds for water purification, molecular sieves (only molecules of certain sizes and shapes can pass through), catalysts, sorbents, gas separator, nitrogen remover in On-Board Oxygen Generating Systems, radioactive material management, solar thermal collectors, ammonia and nitrogenous filter, and soil treatment. For example, in Lake Tahoe, Virginia, Chernobyl accident in Trans-Carpathia area where zeolites/clinoptilolite were applied to treat wastewater and remove radioactive/heavy metals and toxic contaminants (Kallo, 2001; Widiastuti *et al.*, 2008; Petrus and Warchol, 2005; Sprynskyy *et al.*, 2006; Pitcher *et al*., 2004; Misaelides and Godelitsas, 1995; White *et al.*, 1995).

5.1.1.4 Activated Carbon

Activated carbon is an exceedingly permeable, amorphous solid made up of micro-crystallites within a graphite lattice. It consists of carbonaceous material, including coal (bituminous, sub-bituminous, and lignite), peat, wood or nutshells. Activated carbon is employed for adsorption of organic substances as well as non-polar adsorbents and is commonly employed for treatment of waste gas and wastewater. It is the most widely employed adsorbent since the majority of its chemical (e.g. surface groups) and physical properties (e.g. pore size distribution and surface area) can be adjusted to suit relevant requirements. US Environmental Protection Agency cited activated carbon as one of the best control technologies available. (Derbyshire *et al*., 2001). Its practicality also stems from its large micropore (and sometimes mesopore) volume and subsequent high surface area. (Demirbas, 2008). The disadvantage of activated carbon is its production by thermal or chemical processes at temperatures of 450-1200°C. Such energy intensive processes make it less cost-effective than alternative adsorbents (Griswold, 1941; Crini, 2006) and contributed to the carbon-footprint of processes.

5.1.1.5 Biosorbent/Agro-waste

Biosorbents are abundant and low cost that are derived from natural sustainable sources such as agricultural wastes e.g. tea and coffee processing waste, hazelnut shells, peanut husk, red fir, maple, sawdust, pine bark, palm kernel husk, coconut husk, peanut skins, cotton, corncobs, rice hulls, apple wastes, coffee grounds, bark, wool, olive cake, almond shells, cactus leaves, charcoal, banana and orange peels, palm fruit bunch, maize leaf (Demirbas, 2008; Hu *et al.*, 2002; Marshall and Champagne, 1995). Biosorbents have been shown to be alternatives to the existing technologies and are effective in the removal and recovery of contaminants such as heavy metals, pesticides, and organic compounds from the environment (Demirbas, 2008; Volesky and Holan., 1995; Holan and Volesky, 1994; Lee and Volesky, 1997; Kratochvil *et al.*, 1998; Kratochvil and Volesky, 1998; Basso *et al.*, 2002; Pagnanelli *et al*., 2003; Volesky, 2003; Shin and Rowell, 2005; Park *et al*., 2006; Basha and Murthy, 2007; Fogarty *et al*., 1999). Mechanisms involved in the biosorption process include chemisorption, complexation, adsorption-complexation on surface and pores, ion-exchange, microprecipitation, heavy metal hydroxide condensation onto the biosurface, and surface adsorption (Demirbas, 2008; Gardea-Torresdey, *et al*., 2004; Volesky, 2001; Brown *et al*., 2000). Several researchers have demonstrated that different biosorbents immobilise different types of contaminants. For example, kenaf was most effective at removing cadmium ion and alfalfa was most efficient at removing cadmium, copper and zinc ions from treat industrial waste effluent. Spent tea leaves have been shown to have the potential to removal basic dyes from aqueous solutions (Demirbas, 2008; Hameed, 2009). The main advantage of biosorbents are low-cost of the approach, high efficiency, minimisation of chemical and/or biological sludge, regeneration of biosorbent, no additional nutrient requirement, and the possibility of metal recovery (Demirbas, 2008; Ahalya *et al*., 2003; Hu *et al*., 2002). Their efficiency does not rely on biological metabolism making them an ideal pre-treatment for subsequent bio-processes. Hence, the

utilisation of such waste is most desirable (Hameed, 2009). The study of biosorption can be significant from an environmental point of view. Pollutants from wastewater can be removed employing this as alternative technique (Demirbas, 2008; Veglio and Beolchini, 1997).

Figure 5.1: Different immobilisation agents

Nano-Iron (top left), Zeolite (top middle), Bentonite Clay (top right), Activated Carbon (bottom left), Kaffir Lime Leaf (bottom right)

(Nano Material, 2012; Pure nature cures, 2014; Mael, 2012; Northern Filter Media, 2013; Pollen Ranch, 2013)

5.1.2 Aims and objectives

The aim of this set of experiments was to enhance the biodegradation potential of MWFs at the end-of-life by immobilising the toxic components of the chemical mixtures. As shown in Chapter 4, MWF are designed to be biologically resistant to prevent colonisation and biodeterioration in use. Thus, biological treatments alone were only partially effective (as shown in Chapter 4). This could be due to the toxicity as a whole or the presence of "bottleneck" compounds in the metabolic breakdown pathways or indeed the presence of a specific and particularly toxic constituents. Therefore, to reduce toxicity, immobilisation

agents were introduced to the MWFs at the end-of-life as part of a hybrid treatment combining adsorption and bioprocessing. Hybrid technology or immobilisation agents were introduced to examine any differences in degradation pathway relative to bioprocessing only. The rationale was that by immobilising the toxic components of the MWF reduces toxicity and enables more complete biodegradation. The chemistry was examined using Liquid Chromatography in parallel with COD, Nitrate, and Ammonium measurements to gain a detailed insight into which metabolic pathways were impacted.

5.2 Materials and methods

This experiment was designed to screen and identify the most effective immobilising agent in terms of removal of toxicity of the MWF solution. This was measured by toxicity and post-exposure recovery tests (see section 3.2.6 and 3.2.7). Water controls were used as an indication of how toxic the material was on its own. This was done by measuring toxicity and post-exposure recovery (see section 3.2.6 and 3.2.7) of the biosensor exposed in water that contained immobilisation agent. The immobilisation agents were then tested with a single compound B10 (neodecanoic acid), which was found to be a toxic in the single compound screen and it is one of the components in Syntilo 1601 formula (Table 3.10 and Figure 3.12), and a compound mixture BR2 containing neodecanoic acid and Corrguard LSA 8000, which is a hard (in this context resist to biodegradation) amine-acid combination (Figure 4.4). One consideration was that one adsorbent could remove toxicity from the single compound but may not from the mixture. Further study is required to evaluate and understand the adsorption dynamics and detoxification mechanisms in complex mixtures, with several adsorbents.

5.2.1. Selection of immobilisation agents

Samples of B10 (Neodecanoic acid) and BR2 (10% Neodecanoic acid + 10% LSA8000) (1% v/v) were prepared in conical flasks with the additional of M9 minimal growth media (11.28g/L), Magnesium Sulphate, MgSO4 (0.56g/L), Glucose (5g/L), Sucrose (5g/L). In addition, 1.2% v/v of resuscitated mid-exponential MWF acclimated bacteria from freeze dried stocks (section 3.2.4) was added to each flask as an inoculum. Freeze dried stocks were used to minimise any founder effects that may have occurred when using mixed undefined bacterial communities. Immobilisation agents, namely, Kaffir Lime (*Citrus hystrix*) leaf, Zeolite, Nano-Iron, Clay, Nano-silica, silica, and activated carbon beads were prepared. Kaffir lime leaves were ground into a powder to maximise the surface area available for adsorption. Immobilisation agents in powder form were added to the flasks (5g/L). The flasks were stirred at 35^{o}C and agitated at 90rpm in shaking incubator (Innova 44, New Brunswick Scientific) for 48 hours. Using the ground leaves has been shown to have the potential to reduce the resistance of intrapore diffusion and shaking the sample reduced the resistance of film diffusion (Hu *et al*., 2002). Toxicity and post-exposure recovery assays were then carried out (see section 3.2.6 and 3.2.7).

5.2.2. Hybrid technology of biological treatment and immobilisation in Syntilo 1601 biodegradation process

5.2.2.1. Sample preparation

Modified MWF, Syntilo 1601, was prepared as in section 4.2.2 in nine flasks (see Table 4.1). Bacterial inocula from freeze dried stocks of acclimated bacteria grown in active bioreactor (section 3.2.4) were then introduced to the flasks. Biodegradation was carried out for 4 days, after day 4, immobilisation agents including; kaffir lime leaf, and nano-iron,

were selected as candidates for this experiment as they were found to have best results from immobilisation agent optimisation experiment (see section 5.3.1). They were added to individual flasks in triplicate, leaving three flasks as controls. The immobilisation agents were left shaking in a solution containing the MWF at 5% v/v at 90 rpm in the flask for 3 days. The chemical analysis and measurement of COD, Nitrate, and Ammonium were analysed during the experiment. The timeline of the experiment is shown in Figure 5.2.

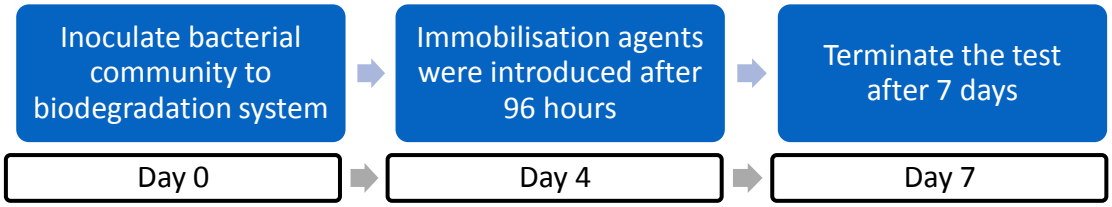


Figure 5.2: Timeline of immobilisation experiment

5.2.2.2. Chemical analysis (LC) / Hach lange kits

Chemical "bottleneck" compound removal by immobilisation was monitored by chemical analysis and COD/Nitrate/Ammonium measurements employing LC and Hach Lange Kits, respectively as per section 4.2.3.1 and 4.2.3.2.

5.3 Results and discussions

5.3.1. Toxicity immobilising agent optimisation

In this study, Figure 5.3 shows the properties of each immobilisation material in reducing microbial inhibition. It was found that Kaffir Lime Leaf and Nano-Iron were the two best immobilisation agents that recovered microbial community from both sample B10 and BR2 compared to the control. Although, zeolite, clay, nano-silica, and silica allowed

biosensor to recover from the toxicity exposure of binary mixture, BR2, it did not recover for compound B10. In contrast, activated carbon bead allowed reduction of bacterial inhibitory of single compound, B10, but not in the binary mixture, BR2. Therefore, nano-iron and kaffir lime leaf were selected for use during the rest of the hybrid treatment work in this Chapter. It has previously been reported that from demonstrations in both laboratory and field scale tests, that iron oxide has shown potential in treating contaminated wastewater (White *et al*., 2009; Girginova *et al.*, 2010) via two paths; adsorption and conversion of the samples to less toxic products (Xu *et al*., 2012). Girginova *et al*., (2010) has shown that silica coated magnetite particles enabled the removal 74% of Hg^{2+} present in water, which suggested exploitation potential to remove heavy metal ions from polluted water.

Figure 5.4 shows an example of the immobilisation treatment of compound B10 (neodecanoic acid). Compound B10 on its own was demonstrated to be toxic as shown in Figure 3.12, Figure 5.3, and Figure 5.4. As shown in Figure 5.4, no bacterial colonies were observed in post-exposure recovery test, indicating the compound was lethally toxic. However, with nano-iron treatment, it can be seen that bacterial colonies were able to recovered on the agar plate after chemical exposure unlike in zeolite treatment. This suggested that nano-iron immobilised or reduced the toxicity of B10 and allowed bacteria to regrow but zeolite was unable to buffer the toxicity (Figure 5.3). The difference in immobilisation efficiency here may have been due to the zeolite and nano-iron interacting on the neodecanoic acid differently. At the pH used in this experiment, the charge of the iron oxide was positive (Sumner, 1963) and it is possible this could have adsorb negatively charged compounds such as neodecanoic acid, whilst the negatively charged zeolite should repel them so was unable to buffer toxicity.

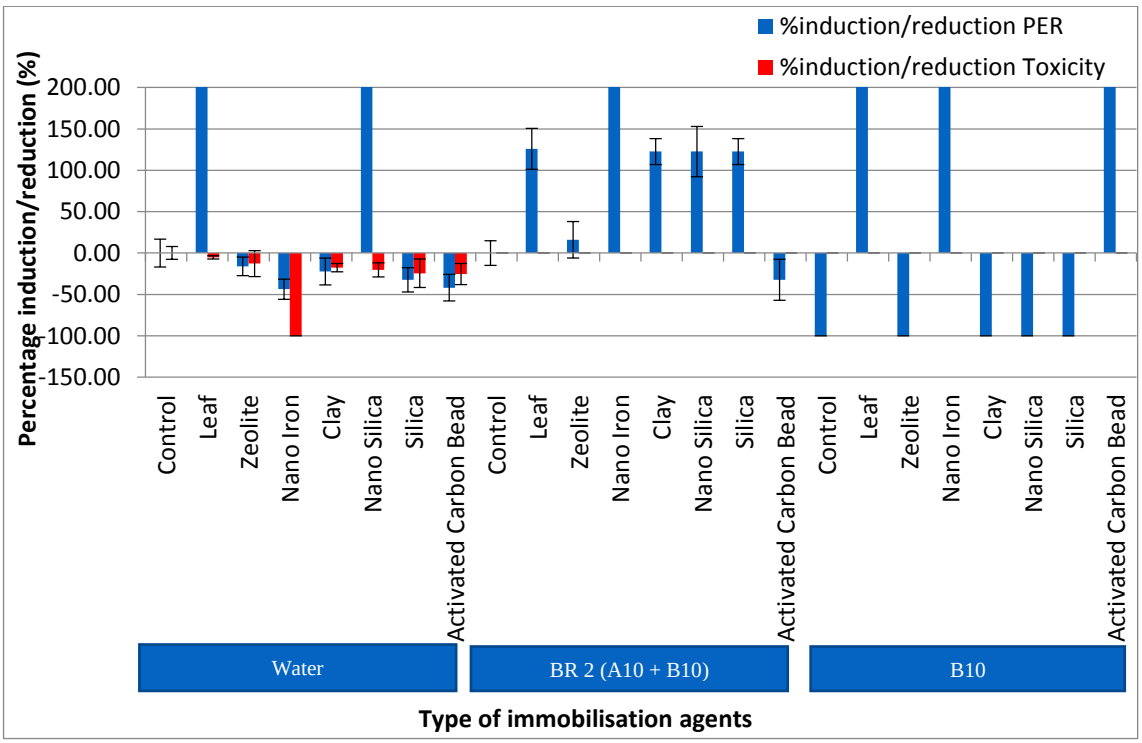


Figure 5.3: The capacity of each immobilisation material in solutions containing BR2, which consisted of A10+B10 and compound B10 on its own. Toxicity and post-exposure recovery of *E.coli* HB101 was tested. Tests were triplicated. Error bars represent the standard deviation of the mean *(n=3).* Note that the toxicity results are shown as 0 due to toxic response to biosensor *E.coli* HB101.

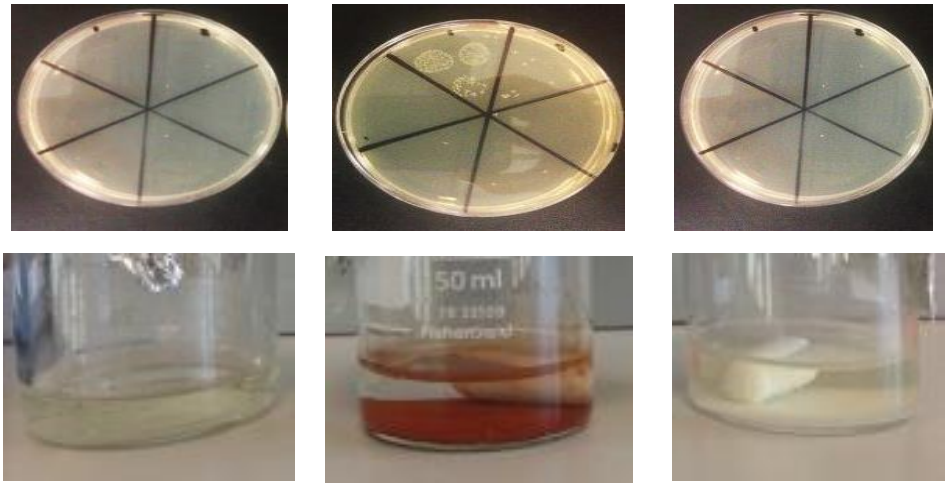


Figure 5.4: Examples of post-exposure recovery results for compound B10 - control (left), B10 treated with nano-iron (middle), and B10 treated with zeolite (right). The treatment period was 2 hours and bacteria community was exposed for 15 minutes. Tests were triplicated.

Immobilisation agents such zeolite, clay, and activated carbon have been reported to perform well in removing toxicity including very toxic waste streams such as organics and heavy metals (lead, nickel, copper) (Hu *et al*., 2002). However in contrast, from this study, they did not perform as well as kaffir lime leaf and nano-iron as can be seen in Figure 5.3. This could have been to the sample types being different to those tested by other researches as majority of research was done on heavy metals and not organics as was the focus of this work. However, several studies focused on organics were in accordance with the findings of this work (Rengaraj, 2002; Hu *et al*., 2002; Xu *et al*., 2012; Iram *et al*., 2010). For example, Iram *et al*. (2010) has shown that Fe_3O_4 hollow nanospheres were effective is adsorbing red dye with the capacity of 90 mg/g.

From the literature review of previous studies, it can be seen that different immobilisation agents adsorb different chemical components or functional groups. For instance, Hu *et al.* (2002) reported similar results, where leaves had similar adsorptive capacity to activated carbon though, it has much larger surface area compared to leaf particle. Hu (2002) stated that "tree leaves were efficient in wastewater treatment by removing pollutants especially metal ions as long as sufficient surface area was supplied. Decreased leaf size not only increases the surface area of the adsorbent unit, more importantly, exposes the intrapores and reduces the mass transfer resistance of intrapore diffusion. Both are positive in terms of enhancing in terms of enhancing adsorption". Rengaraj *et al*., (2002) showed that palm seed coats adsorbed 10-60ppm phenol from a simulated industrial waste. The adsorption capacity was dependent on particle size, i.e. surface area and pH. Interestingly its adsorption capacity was found to be significantly greater than for commercial activated carbon. Activated carbon was shown to adsorb effectively as single compounds but not in mixtures, in this case BR2 which consisted of neodecanoic acid and Corrguard LSA 8000. In contrast, clay, nano-silica, and silica were effective in adsorbing compounds toxicity

when present as a mixture (BR2) but as the single compound (B10 – neodecanoic acid). Only nano-iron and kaffir lime leaf showed positive results both in terms of single compound (B10) and binary mixture (BR2). From the literature, the mechanisms for such results could be via physical adsorption, ion-exchange, chelation, or formation of complexes with the functional groups of natural organic matter (Hu *et al*., 2002; Hameed, 2009; Hameed and El-Khaiary, 2008; Hamdaoui, 2006). However, the mechanism of adsorption of chemical by tree leaves still remains unclear (Hu *et al*., 2002; Marshall and Champagne, 1995).

5.3.2. Biodegradation of Syntilo 1601 in hybrid treatment

Syntilo 1601 is a modified full synthetic MWF developed as described in Chapter 3 (composed of compounds A12 - Triethanolamine, B9 – isononanoic acid, B10 – neodecanoic acid, B30 – Cobratect TT50S, and B32 – Pluronic 17R40). Synthetic MWFs are generally very difficult to degrade biologically, as shown in Chapter 4 of this thesis; and Connolly *et al.* (2006). This is consistent with the known properties of synthetic MWFs relative to semi-synthetics on the hardness in terms of biodegradability (Rossmore, 1989). Hybrid technology is required to remove recalcitrant compounds or to reduce toxicity to aid further biodegradation. Therefore, those selected immobilisation agents (see Section 5.3.1), namely, kaffir lime leaf and nano-iron, were added to flasks after 4 days and changes were observed.

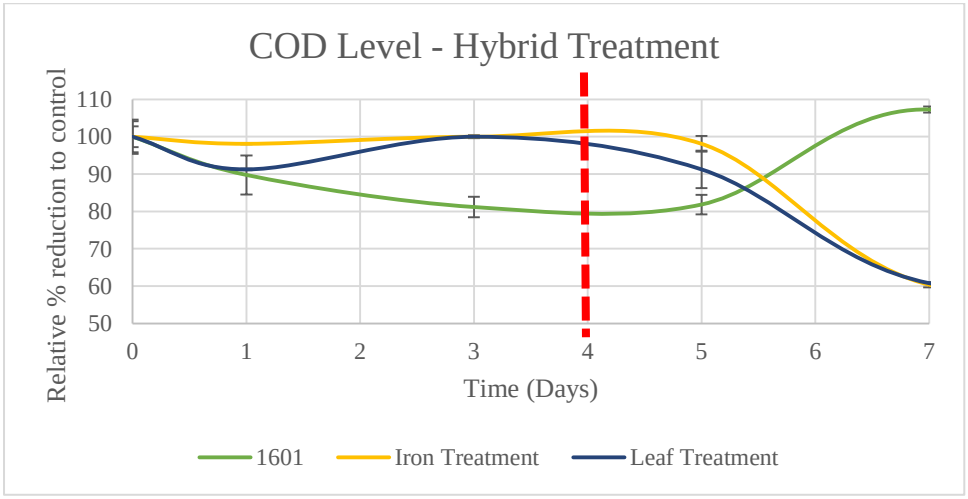
Syntilo 1601 was processed in an aerobic biodegradation reactor for 4 days. No change in the COD reduction was observed. However, at the end of day 4, immobilisation agents; nano-iron and leaf, were introduced to the system and a drop of approximately 40% in

COD was observed from day 5 (see Figure 5.5a). After day 4, COD reduction was significant for both nano-iron ($P < 0.01$) and kaffir lime leaf ($P < 0.01$). This could have been due to the fact that nano-iron and kaffir lime leaf had adsorbed recalcitrant compounds and so facilitated further MWF degradation by the bacterial community. However, there was no significant difference between nano-iron and kaffir lime leaf at most time points.

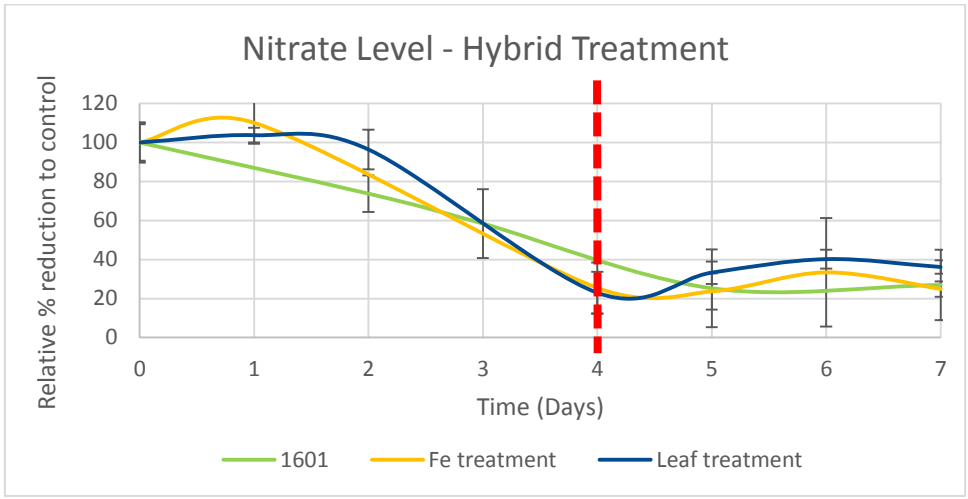
The amount of nitrate was also measured in the MWF. It was observed that immobilisation agents have no impact on the changes in the level of nitrate. Complete N stoichiometry was beyond this study due to resource availability. However, it was assumed that nitrate was converted into ammonium as the level of nitrate fell after day 2, in the same time level of ammonium observed increased (Figure 5.5b and c). This is consistent with Knapp *et al.* (1996).

In addition, the amount of ammonium was also tested. The results in Figure 5.5c, show that ammonium levels in the controls remained constant indicating that biological removal had ceased, whereas, nano-iron and leaf treatment suppressed the production of ammonium compared to the control. This could also been due to the immobilisation agents having removed the amine compound or amine metabolic breakdown products in TCA cycle (see section 4.3.2 and Figure 4.6). Although, Widiastuti *et al.* (2008) has reported that natural zeolites have the greatest potential in removing ammonium but iron and leaf have shown ability to removed ammonium relative to the control. This has shown that biological-adsorbent approach was effective in removing residual ammonium.

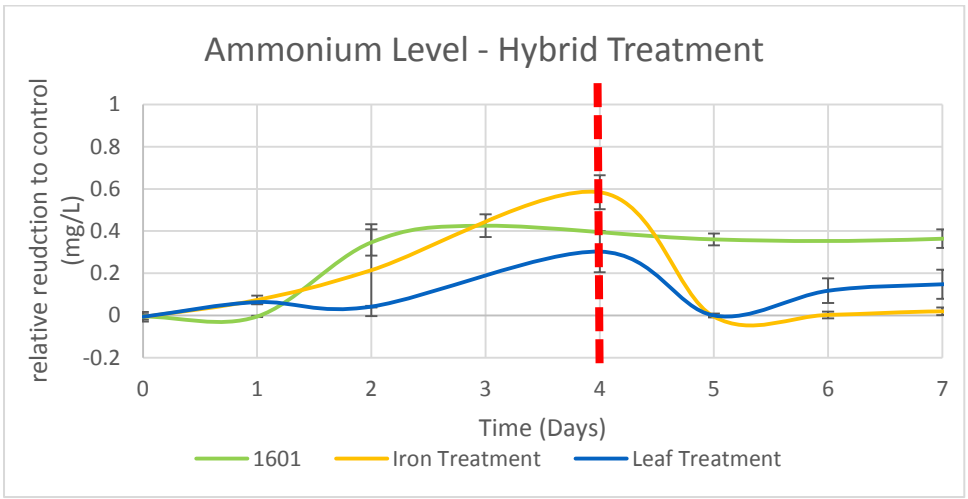
a).



b).



c).



immobilisation agent introduced to the system

Figure 5.5: changes in (a) COD, (b) Nitrate, and (c) Ammonium during the hybrid treatment (Day 0-4 of biodegradation and Day 5-8 of hybrid treatment). Tests were done in triplicate. Error bars represent standard deviation of the mean *(n=3).*

Chromatography has been commonly employed for chemical analysis in various research areas such as medical, forensic, and environmental applications. Liquid chromatography is favoured for soluble compounds. For example, LC is used to detect formation and disappearance of the chemical compounds in Figure 4.3; Rodriguez-Docampo *et al*., 2011; Dong *et al*., 2015. From chemical analysis using LC in this study, many peaks were detected as shown in Figure 5.6, which illustrates the metabolic process. The Figure showed that compound at retention time 10.3 minutes were metabolised by the biodegradation process. Figure 5.6 also showed that kaffir lime leaf removed recalcitrant metabolic by-product at retention time of 11 minutes, which was accumulating. In summary, kaffir lime leaf was able to remove many breakdown product peaks, which those peaks removal were not achieve by biological treatment on its own *i.e.* Degradation profiles (peak composition) for the biological process and the biological process in combination with an immobilisation agent produced contrasting LC profiles. However, there were extra peaks of metabolic compounds or by-products from the immobilisation agent detected from the process. The extra peaks produced from adsorption were not expect to increase toxicity to the solution as Crini (2006) has reported. Dermirbas (2008) has reported that "agricultural by-products usually are composed of lignin and cellulose as major constituents and may also include other polar functional groups of lignin, which includes alcohols, aldehydes, ketones, carboxylic, phenolic and ether groups. These groups have the ability to some extent to bind to heavy metals by donation of an electron pair from these groups to form complexes with the metal ions in solution". This complexation process could explain the immobilisation process taking place.

For nano-iron treatment experiment, similar results to leaf treatment were observed (see Appendix IV). The possible mechanism of organic adsorption could be via surface exchange reactions that occurred until the surface functional sites are fully occupied, and

thereafter contaminants could diffuse into adsorbent for further interactions with functional groups (Xu *et al*., 2012; Ma *et al*., 2005; Zhao *et al*., 2010; Hu *et al*., 2011).

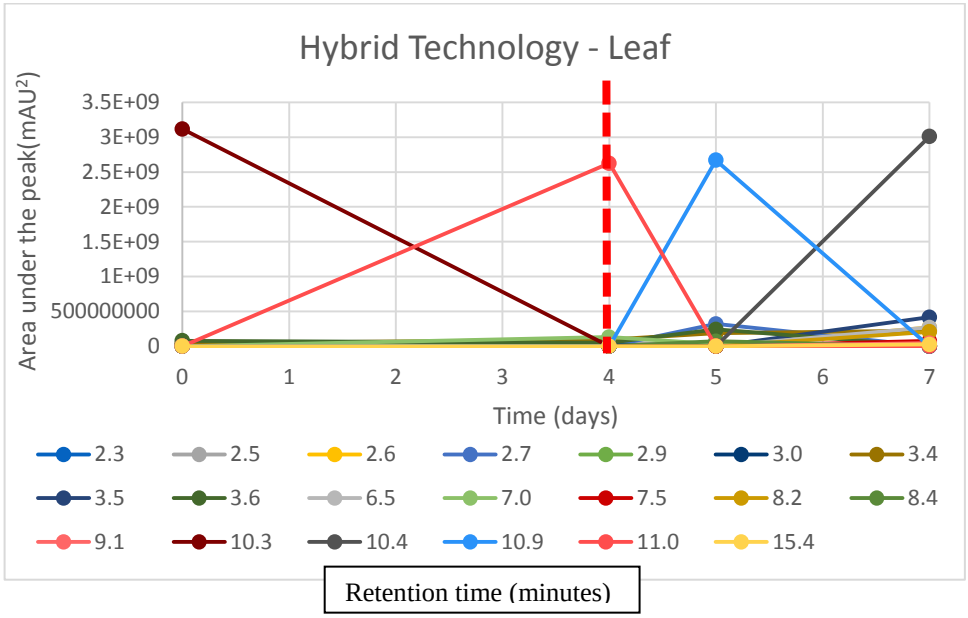


Figure 5.6: Example of chemical analysis by LC in Leaf hybrid treatment process at different retention times from day 0 to day 7. The data represent area under the peaks versus time.

Table 5.1: This table shows the formation and disappearance of peaks after 7 days bioprocessing of Syntilo 1601 from the analysis employing Liquid Chromatography, peaks were identified by retention time. Y indicates yes, N indicates no, empty box indicate no identification of peak at that retention time, and red boxes indicate the peaks observed at T=0.

Retention time (minutes)	2.3	2.4	2.5	2.6	2.7	2.9	3.0	3.4	3.5	3.6	4.2	4.4	6.5	6.8	7.0	7.1	7.5	8.0	8.2	8.3	8.4	8.6	9.1	9.4	9.5	9.6	9.7	10.3	10.4	10.6	10.7	10.8	10.9	11.0	11.1	15.4	15.6	15.7	Total number of peak appeared/ disappeared
Peaks detected at time 0																																							5
Did it biodegrade?	Y		Y					N		Y																		Y											4
Production of recalcitrance compound?		Y		Y					Y			Y					Y	Y						Y						Y					Y		Y		10
Did Fe remove recalcitrance instantly?			N	Y	N	Y	N	N	Y	N				Y	N		Y	Y		Y		Y							Y		Y		N					Y	11
Did Fe remove recalcitrance permanently?		Y	Y	Y	Y	Y	Y	Y	N	Y	N	N			Y		Y	Y						N					Y				Y		Y		Y	15
By product from Fe treatment?																									Y	Y	Y												3
Did Leaf remove recalcitrance instantly?				Y	N	Y	Y	N	Y	N				Y	N		Y	Y		Y	N	Y							Y		Y		N					Y	12
Did Leaf remove recalcitrance permanently?		Y		N	Y	Y		N	N	Y		Y			Y		N	Y			Y			Y						Y				Y		Y		Y	13
By product from Leaf treatment?													Y						Y				Y						Y							Y			5

Table 5.1 shows the summary of the difference in the metabolism and catabolism of Syntilo 1601 with and without the immobilisation agents; kaffir lime leaf and nano-iron. From the Table, the last column is the total number of peaks that appeared or disappeared. For example, there were 4 peaks disappeared from biodegradation process of Syntilo 1601 but 10 new peaks, which were the by-products of catabolic activity of Syntilo 1601. However, with kaffir lime leaf and nano-iron, it was found that 13 and 15 peaks were removed, respectively. Although, there were formation of 5 and 3 new peaks, respectively, as a product of hybrid technologies. To our knowledge, there is still lack of research in this area (see future wok section 6.2.3), with additional of MS, a greater detail insight knowledge could be gained.

5.4 Conclusion

Synthetic MWF, Syntilo 1601 is known to be difficult to biodegrade as demonstrated in Chapter 4. Therefore, immobilisation approaches were evaluated in order to test the potential to improve the efficiency of the biodegradation process. Results from studies detailed in this chapter are in accord with several reports that show that organic waste products can be effective at removing the toxicity of components in wastewaters. Here, the immobilisation agents, kaffir lime leaf, and nano-iron were found to be the most effective in terms of removing toxicity (Figure 5.3). These were applied to biodegradation process aiming to remove “bottleneck” compound and/or reduce toxicity of the mixture. From the immobilisation studies, it was found that nano-iron is slightly more effective than kaffir lime leaf in terms of removal of the recalcitrant peaks (Table 5.1) and similar in terms of reducing COD level of the effluent (Figure 5.5). More work is required to optimise the addition of adsorbents as it was found that kaffir lime leaf and nano-iron were removing different “bottleneck” compounds/by-products. This was in agreement with the literature (Ahuluwalia and Goyal, 2005). Hence, this suggests that the combination of toxicity

immobilisation agent could be used to achieve more effective results in regard of treatment of wastewaters. For example, Srisukh *et al*. (2006) combined kaffir lime oil with guava leaves to improve antibacterial performance. Guava leaves and water extracted from leaves have antimicrobial properties against *Staphylococcus aureus, Sarcina lutea, Mycobacterium phei*, and *Shigella dysenteriae*. In addition, alcohol extracted from kaffir lime has antibacterial property again *Staphylococcus aureus* and *Bacillus subtilis* (Srisukh *et al*., 2006). A lot of research has also shown potential in modifying the binding site of the adsorbent in order to attracted specific pollutants and functional groups (Xu *et al*., 2012). For example, phosphate, carboxyl, amine, and amide groups have been shown to provide adsorption sites for organics and metals, however, this is still not fully understood. Xu *et al*., (2012) reported that modification of iron oxide at nano-scale has the potential to react with different functional groups such as phosphonic acids, carboxylic acid, and amine. The modified NMs seem to avoid aggregation in order to allow maximal binding surface as often NMs tend to aggregate in solution (Lin *et al*., 2008; Xu *et al*., 2012). This influenced by the electrostatic and van der Waals interactions (Chen *et al.,* 2007; Xu *et al*., 2012). Much work is still needed to advance knowledge in the enhancement of NMs stability by reducing their surface energy, which limits their large-scale application (Xu *et al*., 2012). The major advantages of employing adsorbents as a hybrid method are low in cost, simple to regenerate, efficient, and environmental friendly (Crini, 2006).

CHAPTER SIX

CONCLUSIONS AND IMPLICATIONS

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CONCLUSIONS AND IMPLICATIONS

6.1 Conclusions and discussions

Resource scarcity such as fresh water is one of the current major global challenges. In response, legislation designed to protect water quality and supply has been strengthened in many countries. For example, the Biocide Directive 98/8/EC, EU Regulation No. 528/2012, and the EU Water Framework Directive (2000/60/EC) have been implemented with the aim "to provide a high level of protection for humans, animals, and the environment" (European Commission, 2015). Hence, pre-treatment before disposing to the environment has become essential for waste processors and for manufacturers, who produce large scale waste stream e.g. automotive, dyeing, and distilling industries. However, wastewater is often toxic and unable to be treated effectively by biological processes. The current wastewater treatment methods such as chemical and physical treatments are cost and energy intensive (Chipasa, 2011; Thompson and van der Gast, 2010; Tchnobanoglous *et al*., 2004). Hence, to lower the cost for the manufacturer and to meet environmental standard, sustainable product design is significant. Many industries are now moving towards sustainable "closed-loop" product designs, where most or all of the components of a product can be reused at the end-of-the-product-life (Chang *et al*., 2014; Sarioglu *et al*., 2014; Lawrence, J., 2013). A sustainable design approach helps to reduce raw material consumption, total waste production, and ensures effective resource recovery end-of-life (U.S. General Services Administration, 2014). For example, a telephone has been designed with plastics that melt in boiling water to release reusable electronic components from within the phone, thus making recycling easy and cost effective (Wickham, 2014). Life Cycle Analysis (LCA) is one of the main concerns for manufacturers, who are under

pressure commercially and from legislation designed with the "polluter pays principle" in mind to encourage efficient resource utilisation. Long lasting products are, therefore, the ultimate goal for both suppliers and consumers. MWF, for example, are designed to be biologically resistant to prolong tool and product life while in operation by adding toxic compounds such as biocide in the formulation (Cheng *et al.,* 2005).

Modern MWF formulations are being increasingly employed due to their advanced performance characteristics, but unfortunately this is having an adverse effect on biological disposal options (Bio-Wise, 2010). Currently, the majority of product designs still lack awareness of the sustainability and the cradle-to-grave approach, thus, the resources are consumed and meeting the fate of scarcity and waste management are an ongoing problem. Hence, much effort has been put into research in order to design sustainable products. One cradle-to-grave product life cycle approach is to formulate products, which are durable in use but can be biodegraded or bio-recycled at the end-of-life. This study, in collaboration with BP-Castrol, therefore, aimed to design a synthetic MWF, which is bioresistant whilst in use but can be treated biologically at the end-of-life. This is of great importance to MWF manufacturers because of the competitive advantage represented by these products. In addition, current disposal methods are not sustainable and therefore not in accordance with corporate responsibilities. Also, MWF is consumed approximately 2 million tonnes per year in global scale (Kline & Company, 2011), which diluted in water. At 5% working concentration this generates 40,000,000 tonnes of MWF. The industries in the UK alone produce approximately 400,000 tonnes per year of spent MWFs (Connolly *et al*., 2006; Bio-Wise, 2000). Due to the limitation of biological treatment, an estimated of £8 - 16 million per year is spent on the cost of disposal. A MWF designed in cradle-to-grave fashion would allow water to be recycled and other materials to be recovered.

Briefly, the work in this Thesis was aimed at designing a cradle-to-grave MWF that was durable in use but was amendable to biological degradation. A combinatorial approach was used to screen component combinations for bactericidal activity (see Chapter 3). This was used as the basis for component substitution experiments to replace key bio-softening components and ultimately derive a bio-stable MWF formulation (see Chapter 4). Using a hybrid approach combining biological pre-treatment followed by exposure to adsorbents and subsequent biological treatment it was shown that biological treatment could be achieved (see Chapter 5).

There were three main objectives addressed in this work: 1. Reverse engineer synthetic MWF and design a more sustainable formulation (Chapter 3), 2. Identification of bottleneck compounds (Chapter 4), and 3. Apply hybrid technology to remove bottleneck compounds and aids further biodegradation (Chapter 5).

In the first experiment (Chapter 3), the approach taken was to adopt a pre-emptive approach and design, a MWF which was not susceptible to premature biodeterioration whilst, in contrast, formulating it so that at end-of-life it is amendable to biotreatment. Components were screened in combinatorial fashion (ignoring order) to determine which compounds had bio-softening or bio-hardening properties (see Chapter3, section 3.3.2.1). This provided a deeper insight that was previously available to BP-Castrol because many component interactions were counter intuitive or non-additive (Chapter 3, section 3.3.2). For example, two non-toxic components Corfree M1 (B3) and Cobratec TT50S (B30) were highly toxic when combined. Also, the combination of toxic compounds Corrguard LSA 8000 (A10) and Neodecanoic acid (B10) were made less toxic in mixture. Factorial design provided a powerful way of investigating the interactions between MWF components in

terms of their biozhardening or biodegradation. This was useful in designing a sustainable product in Chapter 3 that was commercially competitive in term of cost and biologically disposable. Product stability under field conditions is still to be tested by BP-Castrol on a site(s) to be decided.

MWF components and synthetic MWF (Syntilo 9913) were screened singly and in combination to gain in-depth understanding on toxicity and degradability profile. From the single compound screen, it can be categorised 80 generic MWF components into groups: lethally toxic (45%), benign (3.75%), retardant (6.25%), recalcitrant (1.25%), and toxic to *E.coli* HB101 specifically (30%). Then, in combinatorial studies, traditional approach of Ecodesign for Sustainability (DfS) was applied on the existing synthetic MWF product. It was important to do combinatorial experiment as toxicity and degradability cannot be predicted by using single compound data (see Chapter 3, section 3.3.2, Figure 3.15). However, the full factorial of 8 compound combinations would be 40,320 possible combinations, but in this, the order of compound can be ignored, which reduced the possibility down to 255 combinations. The effect of chemical interaction can be synergistic or antagonistic (see Chapter 3, Figure 3.15; Strachan *et al*., 2001; Fernández-Alba *et al.*, 2002; Preston *et al*., 2000). This result disagreed with Strachan (1989), and Backhaus *et al.* (2000), which stated that the data of single data can be treated independently in the mixture. Hence, by doing combinatorial studies, it was shown that two non-toxic compounds can become toxic and thus provide a chemical switch to resist biodeterioration in the formulation. Chazal (1995) and Collier *et al*. (1990) reported similarly that some chemicals are not claimed to be bactericidal, though they may have bactericidal properties. Employing the combinatorial factorial design approach, TEA was identified as a "softening key" in the original formulation, Syntilo 9913 (Chapter 3, section 3.3.2.1, Figure 3.19). To improve the quality of this formulation, A6 (Octylamine) was found to be the best

alternative amine. This was in agreement with Brutto (2011) that amine with 8 – 12 carbon composition with additional of a hydroxyl group would be best in terms of suppressing biodeterioration and corrosion. The key feature of biological membranes is their amphiphilic characteristic, so having hydrophilic group helps partitioning into lipid layers and thus they can cross through the membrane and into the cell (Golec *et al.*, 1989). This alternative formulation was found to be bioresistant without the use of biocide in the formulation. The main advantage of biocide-free formulation is the lower cost and time in registering the product as a biocide, a legal requirement, it can cost $10 million and take over 5 years (Reisch, 2012). However, BP-Castrol advised that octylamine is costly and was not economically sustainable. Hence, this experiment concluded that combination 5.22 (A12, B9, B10, B30, and B32) was found to be the most sustainable and also acceptably cost-effective formulation. Although, there are many publications on sustainable to MWF, such as vegetable oil based MWF (Kuram *et al*., 2010; Shashidhara and Jayaram, 2010; Alves and Oliveira, 2008) these products are widely accepted to be less bio-stable than mineral oil based formulation. Here, the reformulated synthetic MWF can be said to be a significant finding for sustainable MWF product as it is biocide-free formulation and it only compose of five chemical constituents, which follow many principles in Green engineering. Structure Activity Relationship (SAR) can be a useful tool to make prediction based on the chemical structure and physiological responses to the biodegradability of the mixture (see section 3.3.2 and 6.2.2).

In the second experiment (Chapter 4), the modified MWF from first experiment (Syntilo 1601) was observed under biodegradation process. Despite, the limitation of the chemical analysis instrument, from COD measurement it was found that biocide-free Syntilo1601 performed as well as Syntilo 9913 in terms of biological resistant. This agreed with Connolly *et al*. (2006) that synthetic MWF is resistant to biological treatment. Modern

MWF formulations give advance performance characteristics, which is the reason of increased in the popularity in the usage (Bio-Wise, 2010). In modern formulations some components may provide sufficient carbon, nitrogen, phosphorus and other nutrients required for their growth. Many of the trace elements required for microbial growth are present as contaminants in bulk soured petrochemical products. It must be noted that cutting fluids are complex products, and progress is constantly made to incorporate additives that are less degradable in order to prolong the life of these coolants. This is having an adverse effect on the disposal costs as many treatment systems are unable to treat modern synthetic MWF formulations (Bio-Wise, 2010). Although the COD reduction observed from this study was very small suggesting that the MWF is non-biodegradable, many metabolic breakdown products were observed. This suggested that raw materials were biodegraded only to a limited extent and that as the recalcitrant by-products were produced compound peaks were detected by LC. Compounds that accumulated in the system, represented by persistent LC peaks, were assumed to be "bottleneck compounds". These compounds need to be removed or inactivated before biological treatment can proceed further. Chapter 5 focused on techniques to remove, or inactive "bottleneck compounds" to facilitate further biological treatment.

In the final experiment (Chapter 5), hybrid technologies/immobilisation agents were applied to the biological degradation system in order to remove recalcitrant compounds and aid further degradation. The following four conditions must be considered in the selection of wastewater treatment technologies: 1. Treatment flexibility and final efficiency, 2. Reuse of treatment agents, 3. Environmental security and friendliness, and 4. Low cost (Xu *et al*., 2012; Zhang and Fang, 2010; Oller *et al*., 2011). From the experiment detailed in Chapter 5, nano-iron and kaffir lime leaf were effective as immobilisation agents. Nano-

iron and kaffir lime leaf removed 15 and 13 chemical by-products, respectively. Also, COD was reduced by 40% for both technologies.

6.2 Future Work and Recommendations

This study aimed to design a novel MWF formulation that was resistant to microbial attack during its operational life to prolong the product life, whilst being conductive to bio-treatment end-of-life. This work aimed to develop a sustainable approach to MWF formulation. A bio-hard MWF was formulated without the need for biocide. However, several factors can be improved. In addition, due to limitations in availability of analytical instruments, a simple chemical switch was not identified. The presence of accumulating LC peaks supports the hypothesis that bottleneck compounds exist. Subsequent work with hybrid technologies suggested high potential in removing recalcitrant "bottleneck" chemical compounds to aid further biodegradation. Future work would include more detailed chemical analysis using LC-Mass Spectroscopy (LC-MS). The additional information of the mass of the compounds in the accumulating bottleneck peaks would allow comparison with data bases and potential identification to inform removal strategies.

6.2.1. Reverse Engineering Experiment (Chapter 3)

In this study, toxicity and post-exposure recovery were carried out using single bacterial sensor, *E.coli* HB101. However, it would be useful to do post-exposure recovery tests and flow cytometry on the mixed bacterial consortium to measure the lives/death ratio. The current data in this study could be said to be comparing apples with oranges.

6.2.2. Predictive model

A predictive model would be useful in the formulation process by screening out component combinations with undesirable properties. Any predictive model will require a structure activity relationships (SAR) component. The combinatorial factorial design used in this work identified the predominant properties of each component when in combination with other compounds. Several compounds were predominantly softening and some predominantly hardening. Mixture of A12, B9, and D1, and mixture A12, B9, B10, B32 were unreactive (additive reaction). From section 3.3.2, it has shown that the responses of components that do not interact with any of the other components can be described by simple addition of the single compound responses (Figure 3.15). The metrics used were biological reporters. In order to build useful models *in silico* the biological responses need to be linked to the chemical properties. This will require attention from expert chemists and biologists. More details biological insight into the mechanisms of toxicity may be gained using the flow cytometer to measure cell responses such as membrane integrity, glucose metabolism, efflux pumping and membrane polarity.

6.2.3. Immobilisation technology (Chapter 5)

The work in this Thesis does not allow a detailed conclusion to be made as to the mechanism by which immobilisation technology, nano-iron and kaffir lime leaf, contribute toward the reduction of COD. The possibilities are that they adsorb toxic recalcitrant compound and allow bacterial degradation or it could be by others mechanisms such as oxidation. In addition, the availability of MS would make tremendous difference as the metabolic compounds can be identified and can be tackled with appropriate immobilisation

agent. Furthermore, recalcitrant by-products can be great potential candidates as raw material in future MWF formulations.

This experiment was designed as normal fixed-film bubble column biological treatment (day 0 – 4) and followed by hybrid treatment (day 5-7). Differences due to the immobilisation agents being introduced from the day 0 and also for shorter periods of time were identified. Similar to the bottleneck identification study, the additional MS to LC make tremendous difference as the metabolic compounds can be identified and can be tackled with appropriate immobilisation agent. In addition, toxicity and post-exposure recovery tests are suggested to be done in parallel with the chemical analysis. This would allow better understanding on the mechanism of the immobilisation agent toward reduction of COD. This will reveal whether hybrid technologies were removing toxicity/toxic bottleneck compounds or COD reduction was caused by other reactions such as oxidation.

Once the bottleneck compounds can be identified, appropriate hybrid technologies can be applied. Much research has suggested the high adsorbent ability of modified immobilisation agents such as nano-iron. They could be modified to have selective binding sites. Moreover, as adsorption of organic contaminants took place via surface exchange reactions until the functional sites are fully occupied, and thereafter contaminants could diffuse into adsorbent for further interactions with functional groups (Xu *et al*., 2012; Ma *et al*., 2005; Zhao *et al*., 2010; Hu *et al*., 2011). Based on this mechanism, the development of NMs for organic contaminant removal requires surface modification. The modification and chemical treatment of NMs are essential to enhance the target adsorption capability. Furthermore, literature suggested that different adsorbent interact with different type of compound (Ahuluwalia and Goyal, 2005; Srisukh *et al*., 2006; Xu *et al*., 2012), this

implies that employing combination of hybrid technologies offer a greater chance of "bottleneck" compounds removal.

The work from this Thesis is in process of publishing under the topic of "Sustainable Remediation of recalcitrant industrial waste employing waste by-product as immobilisation agents to remove metabolic 'bottleneck' to facilitate biological degradation" in *Chemosphere.*

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APPENDIX

I. Compound code

A1	Monoethanolamine (MEA)
A2	Diisopropanolamin
A3	Diglycolamine
A4	Monoisopropanolamine (M.I.P.A)
A5	L-2-Amino-1-Butanol
A6	Octylamine
A7	Corrguard EXT
A8	AB95
A10	Corrguard LSA 8000
A11	N-methyldiethanolamine (M.D.E.A)
A12	Triethanolamine (TEA)
A14*	Synergex T Plus
A15	Genamin 3920
A16*	Synergex T Plus
A17	N-Octyldiethanolamin
A18	N,N-Dimethylisopropanolamin
A19	2-Dimethylamino-2-methyl-1-propanol (DMAMP-80)
A20	5-Amino-1-pentanol
A21	Butyl Oxy Propyl Amin
A22	Product 7910
A23	Product 7911
B3	Corfree M1
B4	Irgacor L190
B5	Sebacic acid
B6	Dodecandioic acid
B7	Benzoic acid
B8	Caprylic acid
B9	Isononanoic acid
B10	Neodecanoic acid
B11	Becrosan 2128

B12	SOKALAN CP100	C2	Nynas T22
B13	Citric acid	C3	Nexbase 3043
B14	Lactic acid (80% ig)	C4	Durasyn 166
B15*	Diacid 1550	C6	Indopol H300
B19*	Syntilo 9913	C7	Petronate HL/L
B30	Cobratec TT 50S	C8	Synacto 246
B31	Pluronic 17R20	C9	Sylvatal 25/30LT
B32	Pluronic 17R40	C10	Ligacid OW
B33	Breox 75W18000	C11	Prifac 2990
B34	Genapol PN30	C12	Prisorine 3501
B37	Korantin-LUB + KOH	C13	Rizinolsaeure
B38	Lubrophos LP700 + KOH	C14	Emulgin VP 3370V
B42	Neodecanoic acid + LSA 8000	C15	Emulsogen M
B43	Isononanoic acid + LSA 8000	C16	Alkamuls A
B44	Caprylic acid + LSA 8000	C17	Marlox RT 42
B45	Isononanoic acid + AB 95	C18*	Diacid 1550
B46	Lactic acid + Octylamine	C19	Akypo RO 90
B48*	Syntilo 9913 concentrate	C20	Akypo RCO 960
C1	APE Core 100	C21	Synative AC 3499

C22	Hostacor L	C32	Butyl triglycol
C23	Rapeseed oil	C33	HD Ocenol 80/85V
C24	Synactive ES TMP 05	C34	Hexylene glycol
C25	Lubrirob S100	C35	DCHA
C27*	Priolube 1415	C36	Dibutyletanolamine
C28	Synester GY 25	C37	Alusol ABF 10
C29	Ketjenlube 135	C38	Cooledge BI
C30*	Priolube 1415	D1	EBC-1 Flocculant
C31	Protectol PE		

*internal replicate to check the reproducability

II. Compound combination codes

Combination	Combination Code	Compound Combinations
Singleton	1.1	A12
	1.2	B3
	1.3	B9
	1.4	B10
	1.5	B30
	1.6	B31
	1.7	B32
	1.8	D1

Combination	Combination Code	Compound Combinations
2 Compound Combinations	2.1	A12, B3
	2.2	A12, B9
	2.3	A12, B10
	2.4	A12, B30
	2.5	A12, B31
	2.6	A12, B32
	2.7	A12, D1
	2.8	B3, B9
	2.9	B3, B10
	2.10	B3, B30
	2.11	B3, B31
	2.12	B3, B32
	2.13	B3, D1
	2.14	B9, B10

Combination	Combination Code	Compound Combinations
2 Compound Combinations	2.15	B9, B30
	2.16	B9, B31
	2.17	B9, B32
	2.18	B9, D1
	2.19	B10, B30
	2.20	B10, B31
	2.21	B10, B32
	2.22	B10, D1
	2.23	B30, B31
	2.24	B30, B32
	2.25	B30, D1
	2.26	B31, B32
	2.27	B31, D1
	2.28	B32, D1

Combination	Combination Code	Compound Combinations
3 Compound Combinations	3.1	A12, B3, B9
	3.2	A12, B3, B10
	3.3	A12, B3, B30
	3.4	A12, B3, B31
	3.5	A12, B3, B32
	3.6	A12, B3, D1
	3.7	A12, B9, B10
	3.8	A12, B9, B30
	3.9	A12, B9, B31
	3.10	A12, B9, B32
	3.11	A12, B9, D1
	3.12	A12, B10, B30
	3.13	A12, B10, B31
	3.14	A12, B10, B32
	3.15	A12, B10, D1
	3.16	A12, B30, B31
	3.17	A12, B30, B32
	3.18	A12, B30, D1
	3.19	A12, B31, B32
	3.20	A12, B31, D1
	3.21	A12, B32, D1
	3.22	B3, B9, B10
	3.23	B3, B9, B30
	3.24	B3, B9, B31
	3.25	B3, B9, B32
	3.26	B3, B9, D1
	3.27	B3, B10, B30
	3.28	B3, B10, B31

Combination	Combination Code	Compound Combinations
3 Compound Combinations	3.29	B3, B10, B32
	3.30	B3, B30, D1
	3.31	B3, B30, B31
	3.32	B3, B30, B32
	3.33	B3, B30, D1
	3.34	B3, B31, B32
	3.35	B3, B31, D1
	3.36	B3, B32, D1
	3.37	B9, B10, B30
	3.38	B9, B10, B31
	3.39	B9, B10, B32
	3.40	B9, B10, D1
	3.41	B9, B30, B31
	3.42	B9, B30, B32
	3.43	B9, B30, D1
	3.44	B9, B31, B32
	3.45	B9, B31, D1
	3.46	B9, B32, D1
	3.47	B10, B30, B31
	3.48	B10, B30, B32
	3.49	B10, 30, D1
	3.50	B10, B31, B32
	3.51	B10, B31, D1
	3.52	B10, B32, D1
	3.53	B30, B31, B32
	3.54	B30, B31, D1
	3.55	B30, B32, D1
	3.56	B31, B32, D1

Combination	Combination Code	Compound Combinations
4 Compound Combinations	4.1	A12, B3, B9, B10
	4.2	A12, B3, B9, B30
	4.3	A12, B3, B9, B31
	4.4	A12, B3, B9, B32
	4.5	A12, B3, B9, D1
	4.6	A12, B3, B10, B30
	4.7	A12, B3, B10, B31
	4.8	A12, B3, B10, B32
	4.9	A12, B3, B10, D1
	4.10	A12, B3, B30, B31
	4.11	A12, B3, B30, B32
	4.12	A12, B3, B30, D1
	4.13	A12, B3, B31, B32
	4.14	A12, B3, B31, D1
	4.15	A12, B3, B32, D1
	4.16	A12, B3, B10, B30
	4.17	A12, B9, B10, B31
	4.18	A12, B9, B10, B32
	4.19	A12, B9, B10, D1
	4.20	A12, B9, B30, B31
	4.21	A12, B9, B30, B32
	4.22	A12, B9, B30, D1
	4.23	A12, B9, B31, B32
	4.24	A12, B9, B31, D1
	4.25	A12, B9, B32, D1
	4.26	A12, B10, B30, B31
	4.27	A12, B10, B30, B32
	4.28	A12, B10, B30, D1
	4.29	A12, B10, B31, B32
	4.30	A12, B10, B31, D1
	4.31	A12, B10, B32, D1
	4.32	A12, B30, B31, B32
	4.33	A12, B30, B31, D1
	4.34	A12, B30, B32, D1
	4.35	A12, B31, B32, D1

Combination	Combination Code	Compound Combinations
4 Compound Combinations	4.36	B3, B9, B10, B30
	4.37	B3, B9, B10, B31
	4.38	B3, B9, B10, B32
	4.39	B3, B9, B10, D1
	4.40	B3, B9, B30, B31
	4.41	B3, B9, B30, B32
	4.42	B3, B9, B30, D1
	4.43	B3, B9, B31, B32
	4.44	B3, B9, B31, D1
	4.45	B3, B9, B32, D1
	4.46	B3, B10, B30, B31
	4.47	B3, B10, 30, 32
	4.48	B3, B10, 30, D1
	4.49	B3, B10, B31, B32
	4.50	B3, B10, B31, D1
	4.51	B3, B10, B32, D1
	4.52	B3, B30, B31, B32
	4.53	B3, B30, B31, D1
	4.54	B3, B30, B32, D1
	4.55	B3, B31, B32, D1
	4.56	B9, B10, B30, B31
	4.57	B9, B10, B30, B32
	4.58	B9, B10, B30, D1
	4.59	B9, B10, B31, B32
	4.60	B9, B10, B31, D1
	4.61	B9, B10, B32, D1
	4.62	B9, B30, B31, B32
	4.63	B9, B30, B31, D1
	4.64	B9, B30, B32, D1
	4.65	B9, B31, B32, D1
	4.66	B10, B30, B31, B32
	4.67	B10, B30, B31, D1
	4.68	B10, B30, B32, D1
	4.69	B10, B31, B32, D1
	4.70	B30, B31, B32, D1

Combination	Combination Code	Compound Combinations
5 Compound Combinations	5.1	A12, B3, B9, B10, B30
	5.2	A12, B3, B9, B10, B31
	5.3	A12, B3, B9, B10, B32
	5.4	A12, B3, B9, B10, D1
	5.5	A12, B3, B9, B30, B31
	5.6	A12, B3, B9, B30, B32
	5.7	A12, B3, B9, B30, D1
	5.8	A12, B3, B9, B31, B32
	5.9	A12, B3, B9, B31,D1
	5.10	A12, B3, B9, B32, D1
	5.11	A12, B3, B10, B30, B31
	5.12	A12, B3, B10, B30, B32
	5.13	A12, B3, B10, B30, D1
	5.14	A12, B3, B10, B31, B32
	5.15	A12, B3, B10, B31, D1
	5.16	A12, B3, B10, B32, D1
	5.17	A12, B3, B30, B31, B32
	5.18	A12, B3, B30, B31, D1
	5.19	A12, B3, B30, B32, D1
	5.20	A12, B3, B31, B32, D1
	5.21	A12, B9, B10, B30, B31
	5.22	A12, B9, B10, B30, B32
	5.23	A12, B9, B10, B30, D1
	5.24	A12, B9, B10, B31, B32
	5.25	A12, B9, B10, B31, D1
	5.26	A12, B9, B10, B32, D1
	5.27	A12, B9, B30, B31, B32
	5.28	A12, B9, B30, B31, D1

Combination	Combination Code	Compound Combinations
5 Compound Combinations	5.29	A12, B9, B30, B32, D1
	5.30	A12, B9, B31, B32, D1
	5.31	A12, B10, B30, B31, B32
	5.32	A12, B10, B30, B31, D1
	5.33	A12, B10, B30, B32, D1
	5.34	A12, B10, B31, B32, D1
	5.35	A12, B30, B31, B32, D1
	5.36	B3, B9, B10, B30, B31
	5.37	B3, B9, B10, B30, B32
	5.38	B3, B9, B10, B30, D1
	5.39	B3, B9, B10, B31, B32
	5.40	B3, B9, B10, B31, D1
	5.41	B3, B9, B10, B32, D1
	5.42	B3, B9, B30, B31, B32
	5.43	B3, B9, B30, B31, D1
	5.44	B3, B9, B30, B32, D1
	5.45	B3, B9, B31, B32, D1
	5.46	B3, B10, B30, B31, B32
	5.47	B3, B10, B30, B31, D1
	5.48	B3, B10, B30, B32, D1
	5.49	B3, B10, B31, B32, D1
	5.50	B3, B30, B31, B32, D1
	5.51	B9, B10, B30, B31, B32
	5.52	B9, B10, B30, B31, D1
	5.53	B9, B10, B30, B32, D1
	5.54	B9, B10, B31, B32, D1
	5.55	B9, B30, B31, B32, D1
	5.56	B10, B30, B31, B32, D1

Combination	Combination Code	Compound Combinations
6 Compound Combinations	6.1	A12, B3, B9, B10, B30, B31
	6.2	A12, B3, B9, B10, B30, B32
	6.3	A12, B3, B9, B10, B30, D1
	6.4	A12, B3, B9, B10, B31, B32
	6.5	A12, B3, B9, B10, B31, D1
	6.6	A12, B3, B9, B10, B32, D1
	6.7	A12, B3, B9, B30, B31, B32
	6.8	A12, B3, B9, B30, B31, D1
	6.9	A12, B3, B9, B30, B32, D1
	6.10	A12, B3, B9, B31, B32, D1
	6.11	A12, B3, B10, B30, B31, B32
	6.12	A12, B3, B10, B30, B31, D1
	6.13	A12, B3, B10, B30, B32, D1
	6.14	A12, B3, B10, B31, B32, D1

Combination	Combination Code	Compound Combinations
6 Compound Combinations	6.15	A12, B3, B30, B31, B32, D1
	6.16	A12, B9, B10, B30, B31, B32
	6.17	A12, B9, B10, B30, B31, D1
	6.18	A12, B9, B10, B30, B32, D1
	6.19	A12, B9, B10, B31, B32, D1
	6.20	A12, B9, B30, B31, B32, D1
	6.21	A12, B10, B30, B31, B32, D1
	6.22	B3, B9, B10, B30, B31, B32
	6.23	B3, B9, B10, B30, B31, D1
	6.24	B3, B9, B10, B30, B32, D1
	6.25	B3, B9, B10, B31, B32, D1
	6.26	B3, B9, B30, B31, B32, D1
	6.27	B3, B10, B30, B31, B32, D1
	6.28	B9, B10, B30, B31, B32, D1

Combination	Combination Code	Compound Combinations
7 Compound Combinations	7.1	A12, B3, B9, B10, B30, B31, B32
	7.2	A12, B3, B9, B10, B30, B31, D1
	7.3	A12, B3, B9, B10, B30, B32, D1
	7.4	A12, B3, B9, B10, B31, B32, D1
	7.5	A12, B3, B9, B30, B31, B32, D1
	7.6	A12, B3, B10, B30, B31, B32, D1
	7.7	A12, B9, B10, B30, B31, B32, D1
	7.8	B3, B9, B10, B30, B31, B32, D1

Combination	Combination Code	Compound Combinations
8 Compound Combination (Complete formulation)	8	A12, B3, B9, B10, B30, B31, B32, D1

III. Amine combination codes

Amine Substitution, as TEA (A12) was shown to be softening key in Syntilo product

*A6 Combinations - Syntilo, which does not contain biocide and B3

Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol
A 6.1	ml	A 6.2	ml	A 6.3	ml	A 6.4	ml	A 6.5	ml
H2O		H2O		H2O		H2O		H2O	
A1		**A3**		**A4**		**A8**		**A10**	
B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive
B10		B10		B10		B10		B10	
B30		B30		B30		B30		B30	
B31		B31		B31		B31		B31	
B32		B32		B32		B32		B32	

Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol
A 6.6	ml	A 6.7	ml	A 6.8	ml	A 6.9	ml	A 6.10	ml
H2O		H2O		H2O		H2O		H2O	
A11		**A12**		**A20**		**A22**		**A6**	
B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive	**A10**	
B10		B10		B10		B10		B9	Commercially Sensitive
B30		B30		B30		B30		B10	
B31		B31		B31		B31		B30	
B32		B32		B32		B32		B31	
								B32	

Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol
A 6.11	ml	A 6.12	ml	A 6.13	ml	A 6.14	ml	A 6.15	ml
H2O		H2O		H2O		H2O		H2O	
A6		**A14**		**A14**		**A7**		**A7**	
A12		**A10**		**A12**		**A10**		**A12**	
B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive
B10		B10		B10		B10		B10	
B30		B30		B30		B30		B30	
B31		B31		B31		B31		B31	
B32		B32		B32		B32		B32	

*A7 Combinations - Syntilo, which does not contain B3

Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol
A 7.1	ml	A 7.2	ml	A 7.3	ml	A 7.4	ml	A 7.5	ml
H2O		H2O		H2O		H2O		H2O	
A1		**A3**		**A4**		**A8**		**A10**	
B9		B9		B9		B9		B9	
B10	Commercially Sensitive	B10	Commercially Sensitive	B10	Commercially Sensitive	B10	Commercially Sensitive	B10	Commercially Sensitive
B30		B30		B30		B30		B30	
B31		B31		B31		B31		B31	
B32		B32		B32		B32		B32	
D1		D1		D1		D1		D1	

Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol
A 7.6	ml	A 7.7	ml	A 7.8	ml	A 7.9	ml	A 7.10	ml
H2O		H2O		H2O		H2O		H2O	
A11		**A12**		**A20**		**A22**		**A6**	
B9		B9		B9		B9		**A10**	
B10	Commercially Sensitive	B10	Commercially Sensitive	B10	Commercially Sensitive	B10	Commercially Sensitive	B9	Commercially Sensitive
B30		B30		B30		B30		B10	
B31		B31		B31		B31		B30	
B32		B32		B32		B32		B31	
D1		D1		D1		D1		B32	
								D1	

Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol
A 7.11	ml	A 7.12	ml	A 7.13	ml	A 7.14	ml	A 7.15	ml
H2O		H2O		H2O		H2O		H2O	
A6		**A14**		**A14**		**A7**		**A7**	
A12		**A10**		**A12**		**A10**		**A12**	
B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive
B10		B10		B10		B10		B10	
B30		B30		B30		B30		B30	
B31		B31		B31		B31		B31	
B32		B32		B32		B32		B32	
D1		D1		D1		D1		D1	

Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol
A 8.1	ml	A 8.2	ml	A 8.3	ml	A 8.4	ml	A 8.5	ml
H2O		H2O		H2O		H2O		H2O	
A1		**A3**		**A4**		**A8**		**A10**	
B3		B3		B3		B3		B3	
B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive
B10		B10		B10		B10		B10	
B30		B30		B30		B30		B30	
B31		B31		B31		B31		B31	
B32		B32		B32		B32		B32	
D1		D1		D1		D1		D1	

Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol
A 8.6	ml	A 8.7	ml	A 8.8	ml	A 8.9	ml	A 8.10	ml
H2O		H2O		H2O		H2O		H2O	
A11		**A12**		**A20**		**A22**		**A6**	
B3		B3		B3		B3		**A10**	
B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive	B3	Commercially Sensitive
B10		B10		B10		B10		B9	
B30		B30		B30		B30		B10	
B31		B31		B31		B31		B30	
B32		B32		B32		B32		B31	
D1		D1		D1		D1		B32	
								D1	

Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol	Combination Code	Vol
A 8.11	ml	A 8.12	ml	A 8.13	ml	A 8.14	ml	A 8.15	ml
H2O		H2O		H2O		H2O		H2O	
A6		**A14**		**A14**		**A7**		**A7**	
A12		**A10**		**A12**		**A10**		**A12**	
B3		B3		B3		B3		B3	
B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive	B9	Commercially Sensitive
B10		B10		B10		B10		B10	
B30		B30		B30		B30		B30	
B31		B31		B31		B31		B31	
B32		B32		B32		B32		B32	
D1		D1		D1		D1		D1	

IV. Single compounds ranked by absolute growth

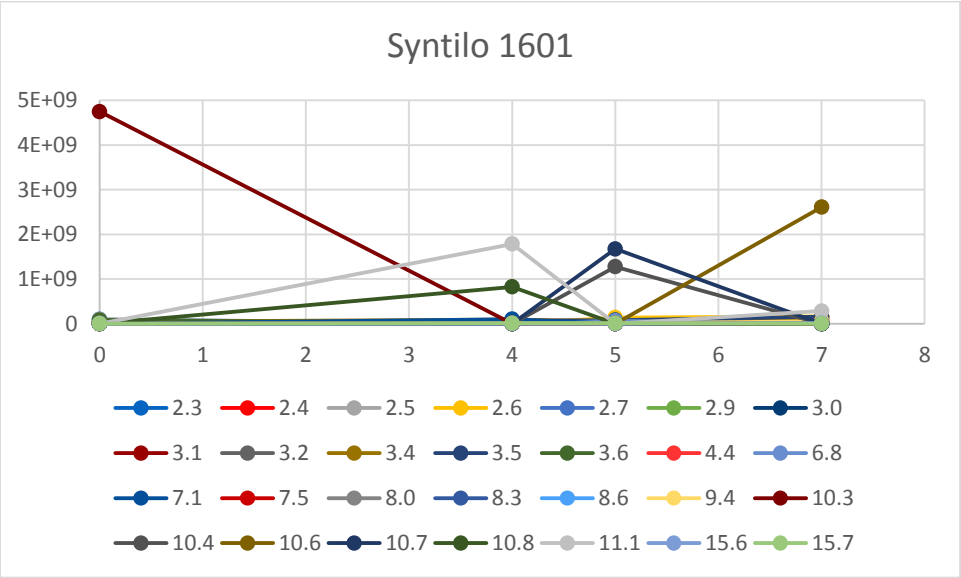
Rank	Combination Code
1	4.56
2	4.67
3	4.47
4	4.66
5	4.69
6	4.60
7	4.50
8	4.59
9	6.28
10	7.8
11	6.27
12	4.51
13	4.68
14	4.57
15	2.4
16	6.15
17	3.28
18	5.28
19	6.24
20	6.19
21	2.21
22	1.1
23	4.61
24	6.14
25	4.63
26	6.21
27	6.20
28	2.2
29	5.41
30	5.56
31	3.53
32	6.11
33	6.17
34	6.12
35	4.48
36	5.37
37	6.25
38	6.23
39	3.1
40	6.16
41	3.6
42	3.3
43	4.38
44	3.54
45	6.26
46	5.22
47	2.10
48	6.10
49	5.49
50	5.53
51	4.13
52	5.35
53	2.14
54	5.47
55	5.29
56	5.54
57	5.45
58	3.27
59	3.47
60	5.46
61	2.18
62	4.53
63	5.48
64	2.7
65	5.55
66	2.20
67	5.26
68	3.8
69	6.4
70	3.50
71	6.18
72	5.38
73	5.52
74	3.41
75	3.49
76	3.26
77	3.30
78	3.48
79	3.42
80	3.22
81	5.13
82	2.19
83	5.34
84	3.52
85	4.43
86	6.8
87	3.40
88	3.43
89	5.50
90	6.22
91	4.22
92	4.37
93	6.7
94	3.10
95	3.38
96	4.28
97	2.1
98	5.44
99	4.15
100	5.51
101	4.12
102	4.36
103	4.19
104	1.4
105	6.5
106	3.33
107	4.39
108	3.18
109	2.22
110	3.24
111	5.23
112	3.29
113	3.46
114	4.16
115	4.46
116	5.32
117	5.33
118	2.13
119	4.52
120	5.30
121	5.43
122	3.39
123	3.45
124	6.6
125	3.56
126	3.37
127	5.4
128	5.1
129	6.13
130	3.21
131	6.2
132	2.23

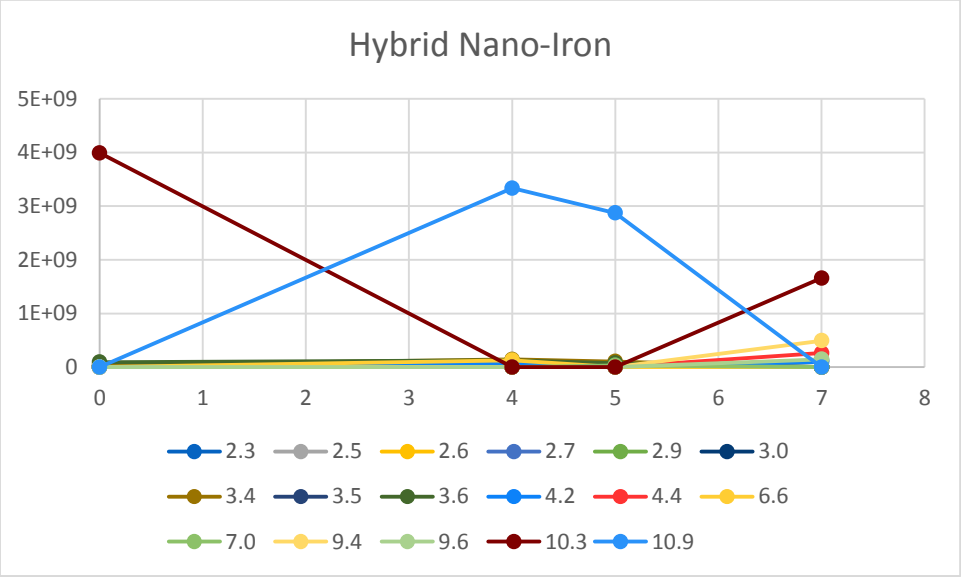
Rank	Combination Code
133	4.9
134	5.3
135	2.25
136	5.7
137	3.35
138	2.9
139	1.8
140	3.4
141	4.58
142	3.51
143	5.6
144	5.11
145	4.31
146	3.44
147	3.11
148	3.17
149	3.31
150	4.24
151	3.36
152	2.28
153	6.9
154	4.55
155	4.1
156	3.55
157	4.42
158	2.15
159	4.64
160	5.42
161	4.2
162	5.21
163	4.25
164	7.3
165	4.8
166	5.24
167	2.11
168	4.54
169	5.36
170	6.3
171	4.62
172	4.49
173	4.65
174	5.10

Rank	Combination Code
175	4.40
176	3.20
177	1.5
178	5.31
179	3.14
180	2.27
181	3.32
182	2.3
183	4.45
184	5.14
185	2.26
186	4.34
187	3.15
188	5.17
189	5.25
190	5.12
191	4.33
192	4.70
193	3.19
194	6.1
195	3.7
196	4.35
197	4.30
198	3.16
199	4.14
200	4.5
201	5.39
202	3.2
203	2.17
204	5.9
205	5.40
206	4.26
207	4.32
208	5.5
209	4.17
210	1.3
211	3.12
212	4.29
213	5.27
214	5.15
215	3.23

Rank	Combination Code
216	1.6
217	5.2
218	7.7
219	3.9
220	3.13
221	4.44
222	5.18
223	2.16
224	4.18
225	4.41
226	5.19
227	4.4
228	5.8
229	2.5
230	7.6
231	7.4
232	3.25
233	4.20
234	5.16
235	8
236	5.20
237	3.34
238	4.6
239	1.7
240	7.2
241	4.7
242	4.21
243	4.27
244	7.5
245	3.5
246	4.23
247	4.10
248	7.1
249	2.8
250	4.3
251	4.11
252	2.24
253	1.2
254	2.6
255	2.12

VI. Complete hybrid data





VII. Poster

A study of the effects of nano-scale iron and zeolite on the toxicity of chemical mixtures employing a rapid assessment method

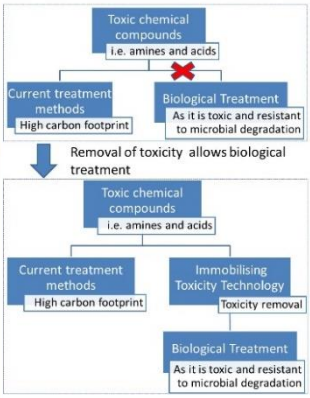
Boontida Uapipatanakul, Duane Ager, Ian P. Thompson

Abstract

The introduction of new industrial products such as detergents, surfactants, and solvents requires assessment to determine the potential environmental impact of their individual chemical constituents. A high throughput toxicology assay has been developed to distinguish the lethal, acute toxicity, and bacteriostatic effects of the soluble constituents. Inhibitory (acute) and lethal effects were determined using complementary techniques of biosensor and post-exposure recovery (colony-forming rate) measures to identify the relative toxicity of the substance. Additionally, adsorptive particles, nano-scale iron and zeolite, were added to assess their potential to buffer the toxicity of test compounds. Nano-scale iron was found to facilitated reduction in the toxicity of the test chemicals to *E.coli* HB101, and allowed bacteria colonies to recover most compared to water control and zeolite.

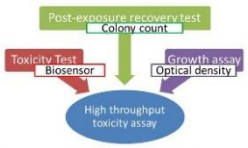
Introduction

Amines and acids are common chemical constituents in many industrial products such as detergents, surfactants, and solvents [1,2].



Both zeolite and iron (hematite) have good adsorption abilities. They have been many studies on adsorption and removal of heavy metals using iron and zeolite[3,4].

The level of toxicity can be assessed by employing a high throughput toxicology assay. This technique allows toxicity and inhibitory effects to be distinguished.



Results

All samples were toxic to both *E.coli* HB101 and mixed communities. Amine -A and organic acid -B were found to be lethally toxic but organic acid -C was found to be inhibitory. After pre-treatment employing adsorptive particles, nano-scale iron and zeolite, the toxicity immobilisation was 100% and 66.6%, respectively.

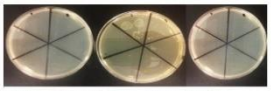


Figure 1: Compound B no-, Fe-, and zeolite-treatments, respectively. Fe-treatment allowed bacterial recovery but zeolite did not.

Figure 2: Compound B no-, Fe-, and zeolite-treatments, respectively. Fe-treatment had an obvious visible change from yellow to clear

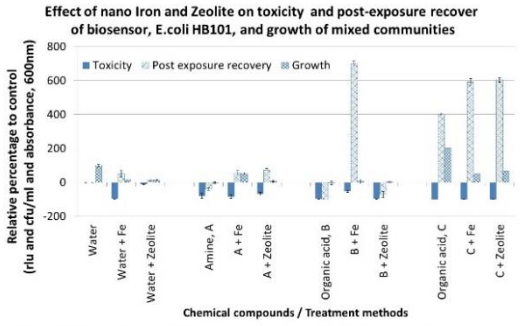


Figure 3: Effects of nano-scale iron and zeolite on toxicity of the compounds, post-exposure recovery of biosensor, *E.coli* HB101, and growth of mixed communities. The error bars represent standard deviation of triplicate samples.

Methods

Sample preparation
- 1% v/v of sample amine-A, and organic acids –B and -C

Toxicity Immobilising technology
- Add 5g/L of iron and zeolite to test samples for 2 hours

High throughput toxicity assay
- Toxicity test: measure luminescence of *E.coli* HB101
- Post-exposure recovery test: colony forming on nutrient agar
- Growth assay: measure optical density of mixed community of bacteria over 24 hours

The tests were done in triplicates

Conclusions and discussions

The triple approach of testing toxicity with biosensors together with post-exposure recovery assessment and degradation by mixed communities enabled an effective assessment of the degree to which each chemical was toxic. This distinguished lethal, acute toxicity, and bacteriostatic effects of chemical constituents. It was found that nano-scale iron facilitated a reduction in toxicity of the substances to *E.coli* HB101, and allowed bacterial colonies to recover better when compared to water control and zeolite.

Hence, pre-treatment hybrid technology allows toxicity to be immobilised and aids biological treatment

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Contact: meg.uapipatanakul@eng.ox.ac.uk
Department of Engineering Science, University of Oxford, Parks Road, Oxford OX1 3PJ, United Kingdom