Anaerobic Treatment of A Metalworking Fluid and Overcoming the Toxic Effects on the Biodegradation Process

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ABSTRACT

Metalworking fluids (MWFs) are petroleum emulsions employed for metal machining processes as coolants and lubricants. To date, they have been irreplaceable in modern heavy and manufacturing industries, with annual usage exceeding two billion litres worldwide. However, the large amount of MWFs, the highly concentrated complex recalcitrant and toxic petroleum components contained in them continue to cause significant concern in terms of sustainable routes of end-of-life treatment and disposal. Compared with other treatment methods, the anaerobic treatment method has significant advantages, such as the low capital, operating and maintenance costs and energy recovery. This latter factor has the potential benefit of generating bio-energy from waste organic matter whilst aerobic route leads to CO_2 emission. However, the bio-toxicity of MWFs is a huge challenge in terms of employing bio-treatment of waste MWFs. In this study, the anaerobic biodegradability of a typical MWF was investigated employing an activated sludge experimental system. Furthermore, the toxic effects of the MWF on the anaerobic ecosystem, particularly on methanogen species, were investigated using bio-molecular analytical methods and a biosensor. In order to overcome its toxicity, the indigenous anaerobic bacteria isolated from spent MWFs were employed in the treatment of the MWF since they were assumed to be acclimated to the conditions. The major findings include: (1) approximately 80% of the MWF (5,000mgCOD/L) was found to be anaerobically biodegradable, with around 35% of the biodegraded COD could be converted to methane; (2) the MWF appeared to be toxic to the anaerobic ecosystem, especially to methanogen species; and (3) however, treatment employing the anaerobic

bacteria successfully reduced the toxicity of the MWF and enhanced the methane production in the process.

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ABBREVIATIONS

ASP	Activated Sludge Process
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
DGGE	Denaturing Gradient Gel Electrophoresis
DI	De-ionised
DTI	Department of Trade and Industry
ECP	Extracellular Polymers
EOP	Electrochemical Oxidation Potential
FISH	Fluorescent in Situ Hybridization
GC	Gas Chromatograph
HRT	Hydraulic Retention Time
MLSS	Mixed-Liquor Suspended Solid
MIC	Minimum Inhibitory Concentration
MSDS	Material Safety Data Sheet
MWF(s)	Metalworking Fluid(s)
OLR	Organic Loading Rate
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
SEM	Scanning Electron Microscope
SMA	Specific Methanogenic Activity
SRB	Sulphate Reducing Bacteria

SSV	Settled Sludge Volume
TCD	Thermal Conductivity Detector
TOC	Total Organic Carbon
TN	Total Nitrogen
TP	Total Phosphorus
UASB	Up-flow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acid
VSS	Volatile Suspend Solid

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Chapter 1

Introduction

Metalworking fluids (MWFs), which are sometimes referred to as metal-cutting fluids, are oil-based and water-based petroleum emulsions employed for metal cutting and machining processes as coolants and lubricants. They also help to prevent surface corrosion of the parts machined and extend the life of machining tools. The history of utilising MWFs dates back to ancient Egypt (BP, 1969), and they continue to play an irreplaceable role in the modern heavy and manufacturing industries. The annual usage of MWFs exceeds two billion litres worldwide. However, the large amount of MWFs has become a great threat to public and environmental health when MWFs become operationally exhausted. This is because MWFs contain highly concentrated complex of recalcitrant and toxic petroleum components which can cause significant contamination to environment and to human health. With the development of MWFs, a wide variety of chemicals including organic and inorganic matters have been used. Furthermore, there is an increasing tendency for manufactures to formulate increasingly recalcitrant products which make the task of sustainable disposal of spent MWFs increasingly challenging.

To date, chemical, physical and biological wastewater treatment methods have been employed in the disposal of spent MWFs since landfill disposal has been prohibited. Physical and chemical methods, such as coagulation, evaporation and membrane filtration, are commonly employed in the disposal of petroleum wastewater like MWFs. However, the physical and chemical methods have their own limitation in term of

efficiency, particularly the requirements for high energy input, consumables and hazardous chemicals as well as high financial costs including capital investment and operating costs (Cheng et al., 2005). Compared with the physical and chemical methods, biological wastewater treatment methods, including aerobic and anaerobic degradation, have significant advantages, such as their low capital, operating and maintenance costs, plus the fact that no hazardous chemical are required (UKLA, 2011).

In recent years, the demand for a cost effective and environmentally friendly treatment method for spent MWFs has markedly increased because of tighter legislations and regulations on environmental protection such as the Water Framework Directive (2006/EC) and the Urban Waste Water Directive (2003 amended regulations). Thus, biological treatment methods are increasingly attractive options, especially the anaerobic treatment method since it has the potential benefit of generating bio-energy from waste organic matter. Compared with the aerobic degradation process, the anaerobic degradation process enables us to convert organic matters in wastewater to biogas instead of producing CO_2 emission. Because of this great feature, the anaerobic treatment of spent MWFs has shown enormous potential.

However, it is a huge challenge to employ anaerobic treatment method in the disposal of MWFs, since they contain highly concentrated organic wastewater composed of various and complex petroleum compounds and toxic matters. In previous studies, it has been found that anaerobic bacteria are very sensitive to toxicity (Kim et al., 2012; Scherr et al., 2012; Sheppard et al., 2005), especially methanogens. Furthermore, compared to studies of aerobic systems, anaerobic processes and communities are technically more complex to undertake (van der Gast, et al., 2001, 2003a, 2003b, 2004; Cheng et al., 2004).

However, the advent of molecular biological methods has certainly aided the investigation into the anaerobic degradation process of MWFs (Green & Sambrook, 2012; Scherr et al., 2012).

The overall aim of this study was to investigate the anaerobic degradation of a typical MWF and investigate the potential to reduce its inhibitory effect on methangenesis in the degradation process using acclimated anaerobic isolates. A typical water-based MWF (RELUBRO 800 BIO) was selected for this study.

This thesis is composed of six chapters including this introduction.

Chapter 2: Literature Review

A literature review of the background and previous studies on MWFs and their disposal methods is given in this chapter. The major content of this chapter include: overview of MWFs; the health, safety, and environmental risks of utilising MWFs; the chemistry of MWFs; an overview of industrial wastewater treatment and relevant policies and regulations; the disposal methods of MWFs; the anaerobic treatment of MWFs, and the microbiology of MWFs.

Chapter 3: Anaerobic Biodegradation of the Metalworking Fluids

The research described in this chapter aimed at investigating the anaerobic biodegradability of the MWF. Flocculent and granular sludge were used in the studies as inocula in a flask-scale reactor system developed for investigating the anaerobic biodegradability of MWFs systematically. The results obtained from the experiments demonstrate that the MWF to be anaerobic biodegradable and methane was produced in

the process. Meanwhile, the results show a significant inhibitory effect of the MWF on the anaerobic degradation process, especially on methane production efficiency.

Chapter 4: Examining the Toxic Effects of the MWF on Methangenesis

The studies described in this chapter were carried out as further analysis of the toxic effects caused by the MWF on methane production in the anaerobic degradation process. The toxic effects on the activity and community of methanogens in anaerobic sludge were investigated. Two molecular biological methods were employed in the experiments, the Fluorescence *In-Situ* Hybridisation probe (FISH) method and the Denaturing Gradient Gel Electrophoresis (DGGE) method. Subsequently, a toxicity analysis method using a bioluminescence-based bacterial biosensor, *E.coli HB101_pUCD607_ lux*, was developed for real-time measurement of the MWFs' toxicity on the anaerobic degradation process. This method was used in further studies described in Chapter 5.

Chapter 5: Overcoming the Toxic Effects of the MWF on the Anaerobic Degradation Process.

In order to overcome the toxic effects of the MWF on its anaerobic degradation process, a method of microbiological enhancement of the anaerobic degradation process was proposed in this study, exploiting the anaerobic bacteria colonising in spent MWFs to treat the MWF to reduce its toxicity in the anaerobic degradation process.

The first section of this chapter concerns the anaerobic bacteria isolation process from various spent MWFs, as well as bacteria selection and identification using genetic fingerprint analysis. In the second section the optimal growth conditions of the anaerobic

isolates were investigated, and a mixed culture was assembled of the isolates which were subsequently employed in the treatment of the MWF for overcoming its toxicity.

Chapter 6: Conclusions and Recommendations

Further discussions regarding the major findings described in thesis given in this final chapter with recommendations for future work.

Chapter 2

Literature Review

2.1 Overview of Metalworking Fluids

Metalworking fluids (MWFs), which are often referred to as metal-cutting fluids in previous studies, are widely used for machining processes as coolant, lubricant and the medium for removing metal chippings. The functions of MWFs in the machining processes also include corrosion resistance, extending the life of machining tools, arresting re-welding, decreasing surface roughness, increasing dimensional accuracy and reducing energy consumption (El Baradie, 1996; Irani et al., 2005; Brinksmeier et al., 1999).

The use of MWFs dates back to ancient Egypt, and they have played an essential role in the modern heavy and manufacturing industries (BP, 1969). The major limitation of MWFs is that they deteriorate as a result of the exposure to high temperatures, stresses and microbial biodegradation in machining operations and ultimately require replacement. The MWFs which are replaced become waste effluents from machining and have to be treated and disposed of in a safe and sustainable manner. It has been reported that annual usage of MWFs has exceeded 38 million metric tons worldwide with a projected increase of 1.2% in every year. Moreover, the amount of diluted waste effluents from MWFs is more than ten times annual usage, because MWFs arrive on-site concentrated and are diluted prior to use (1 in 10) (Cheng et al., 2005; Shashidhara & Jayaram, 2010; Kline &

Company, Inc., 2006). In the European Union, the annual consumption of MWFs is approximately 320,000 tonnes, with at least two thirds of them needing to be disposed of (Lawal et al., 2012).

It has been reported that MWFs create both human health and environmental problems during their life time (Xu, 2007; Cheng et al., 2005). The environmental pollution caused by spent MWFs is mainly due to the presence of complex recalcitrant chemicals and toxic components; while the disposal processes of spent MWFs may also produce solid hazardous wastes and toxic air emissions (Cheng et al., 2005). MWFs are also associated with high costs in their life cycle. It is estimated that 10% to 17% of total metals' manufacturing costs are spent on acquisition, maintenance and disposal of MWFs (Dettmer, 2004; Winter et al., 2012; Klocke & Eisenblätter, 1997; McClure, 2000). In the UK industry, more than 16 million GBP is annually spent on the disposal of spent MWFs, and the costs are increasing because of tighter regulations (Cheng et al., 2005). In order to solve the problems of high environmental risks and costly disposal of spent MWFs, many studies have been carried out on recycling/disposal methods and new formulations of MWFs (Greeley & Rajagopalan, 2004; Cambiella et al., 2007).

2.2 Environmental Impacts of Metalworking Fluids

MWFs are irreplaceable in the modern machining industries and offer considerable benefits for machining processes. Despite the widespread applications of MWFs, however, they have significant safety, health and environment impacts throughout their life cycle.

There are two aspects to the environmental risks of MWFs in their life cycle. The first aspect concerns their impact on the environment around machining operations, such as aerosols, generated odours and leakage, which leads to health and safety issues for the operatives. Exposure to MWFs is associated with respiratory disorders and various cancers (Simpson et al., 2003; Macherer et al., 2003; Stear, 2005; Xu, 2007). Skin contact may cause some skin diseases such as folliculitis, dermatitis, and skin cancer (Sprince et al., 1996; Kalhofer et al., 1997; Calvert et al., 1998). Inhalation of MWF aerosols is associated with some respiratory effects, such as coughing, phlegm and wheezing, as well as occupational airway diseases such as asthma, chronic bronchitis, airway irritation, acute impairment of lung function and hypersensitivity pneumonitis (Bennett & Bennett, 1985; Mattsby-Baktzer et al., 1989; Greaves et al., 1997; Khan et al., 2009; Lillienberg et al., 2010). In addition, the microbes colonising in MWFs also cause healthy and safety problems associated with pathogens, as producers of toxins or as well as catalysts of chemical deterioration (Bennett & Wheeler, 1954; Hill, 1983; Zeman et al., 1995; Moore et al., 2000). The second aspect concerns the impact of their disposal on the environment when they become operationally exhausted, which is introduced in section 2.1 above.

2.3 Chemistry of Metalworking Fluids

It is well known that the components employed in the formulations of MWFs determine their properties and performance, including cooling, friction reduction and reduction of the shear strength of the work materials. However, the chemical components make sustainable disposal of MWF challenging – added to this new formulation are continually being introduced many of which are even more problematic (Shaw, 1986; Cheng et al.,

2005). With the development of machining technologies and applications, there is a huge demand for MWFs with better performances, such as higher thermal conductivity and lower friction. The fulfilment of this demand has promoted the development of new formulations and the employment of new additives. As a consequence, over 300 substances are known to be employed in MWFs including both organic and inorganic components (Rabenstein et al., 2009).

2.3.1 Classification of Metalworking Fluids

In general, MWF emulsions can be categorised into two main types: oil-based MWFs and water-based MWFs. Oil-based MWFs can be classified into straight oil and soluble oils. Similarly, water-based MWFs can be classified into synthetics and semi-synthetics (Foltz, 2002; Blenkowski, 1993).

1) Straight/Neat Oils

Straight/neat oils are oil-based MWFs which normally contain no water; consisting of 60% ~ 100% highly refined mineral oil or reprocessed oils from various sources, such as animal, marine and vegetable oils. Straight oils are non-corrosive and stable, but many of them do not contain additives (Barth, 1986; Anderson et al., 2003). Straight mineral oils do not have very good lubrication properties in the absence of additives, but they cost less; they are restricted to light duty operations where the lubrication and cooling requirements are not extreme.

2) Soluble Oils

Soluble oils are based on mineral or synthetic oils in high concentration (40% ~70%), and they are fundamentally oils blended with emulsifiers. The emulsifiers make the oils mix or dilute with water in a ratio of about 1 to 20 to form an oil-in-water emulsion (MDC, 1980). The emulsifiers can significantly reduce surface tension and break the oils into small particles and keep them dispersed in water.

3) Synthetic MWFs

Synthetic MWFs are a type of water-mix MWFs which contain various synthetic organic compounds instead of mineral oils, such as hydrocarbon based polyaphaoleins and polyethylene glycols. It also can be defined as the mixture of highly soluble organics without any mineral oils. There are many different chemical agents and additives employed in synthetic MWFs such as extreme pressure (EP) agents, corrosion inhibitors, and wetting and blending agents.

4) Semi-synthetic MWFs

Semi-synthetic MWFs combine soluble and synthetic oils with chemical fluids. Typically, semi-synthetic MWFs contain 10% to 55% mineral oil and synthetic organic compounds. Thus, the advantages and limitations of semi-synthetic MWFs are similar to those described for synthetic MWFs, except that the former have better lubricating properties, and semi-synthetic MWFs can offer good lubrication and heat reduction with a longer sump life. They have been widely used in industry for various moderate and heavy duty machining and grinding applications. In addition, semi-synthetic MWFs are also

considered to be cleaner with better rust and rancidity control than oil based MWFs (MDC, 1980).

In recent years, water-based MWFs have been replacing oil-based MWFs in the machining industries because of their better performance and hazard-free compositions. Thus, a typical semi-synthetic MWFs was selected for this study. However, individual water-based MWF can contain up to 60 constituents (Rebenstein et al., 2009); which makes understanding the chemistry of MWFs important in this study.

2.3.2 Compositions of Metalworking Fluids

It was pointed out by Byers (1994), as citied by Cheng (2005), that the substances employed in MWFs can be classified into seven categories according to their functions in MWFs, including base oil, emulsifiers (surfactants), corrosion inhibitors, extreme pressure agents, biocides, and alkaline reserve agents. Some MWFs also contain anti-foaming agents and metal passivators (Napier et al., 1988; Anderson et al., 2003; Cheng et al., 2005).

The formulations of oil-based MWFs and semi-synthetic MWFs are based on mineral oils which are highly refined from crude oils. Mineral oils are not only one of the major components of MWFs, but also provide a base for other additives (Irani et al., 2005; Webster et al., 1995). Water is one of the major components in water-based MWFs; it is an element which has a high specific heat, high thermal conductivity and a high heat of vaporisation. Thus, it is known as one of the best cooling media (El Baradie, 1996).

1) Mineral Oils

Mineral oils are naphthenic and paraffinic hydrocarbons refined from crude oil. The mineral oils used in MWFs are hydrogenated (hydrotreated) so that most of the carcinogenic polycyclic aromatics can be destroyed or neutralised (Irani et al., 2005; Webster et al., 1995; Tucker, 1994).

2) Emulsifiers and Surfactants

Emulsifiers and surfactants are added to water based fluids to break the oils into minute particles that disperse in water, and they can reduce the fluid's surface tension significantly. Petroleum sulphonates and ethoxylate products are commonly employed in water based MWFs. However, water based MWFs may cause foaming problems in operations, particularly when subjected to shearing and turbulence. Foaming problems can be solved with the employment of wetting agents and foam depressants (El Baradie, 1996; Anderson et al., 2003).

3) Corrosion Inhibitors

Corrosion inhibitors are one of the major additives utilised in MWFs, and can create protective films on the surfaces of metals and tools to prevent corrosion. The chemicals used as corrosion inhibitors include fatty acids salts, sulphonates, amines, amides, borates, silicates, phosphates and nitrates (Anderson et al., 2003; Cheng et al., 2005). For example, alkanolamines and boric acid have been widely employed in MWFs (BLF 2003).

4) Extreme Pressure Agents

Extreme pressure (EP) additives are added to MWFs designed for machining operations with particularly high cutting forces, such as tapping and broaching, and operations

performed with heavy feeds (El Baradie, 1996). EP agents can create a protective shear layer against the welding of the work piece in heavy duty machining operations. Phosphate esters and sulphurised esters are mainly used as EP additives in MWFs (Cheng et al., 2005; Anderson et al., 2003).

5) Biocides

Biocides are added to MWFs to inhibit and control the growth of microorganisms such as bacteria, algae and fungi. The literature review reveals that many different compounds have been used in MWFs as biocides. Typically, biocides are metallic, phenolic or chlorinated organic matter; phenolic matter may be used if there is no concern about disposal (El Baradie, 1996; Anderson et al., 2003; Cheng et al., 2005). Other compounds are also used as biocides in MWFs such as formaldehyde (FA)-based industrial biocide and organosulphur nitrogen compounds. (Rossmore, 1981; 1983; Carvalhinha et al., 2010; Gilbert et al., 2010).

6) Alkaline Reserve Agents

The presence of alkaline components makes the typical pH range of MWFs between 8 and 11 so as to control bacterial growth, maintain rust protection, retain emulsion stability, and aid cleaning. Sodium hydroxide and ethanolamines are commonly used as alkaline reserve additives (Perez et al., 2006; Anderson et al., 2003).

The chemicals that are widely used in different types of MWFs are summarised in Table 2.1 below.

Table 2.1: A summary of the chemicals used in the formulations of MWFs (Adapted from Cheng et al, 2005)

Type of MWFs	Composition	References
Synthetic	Ethanolamines, Polyglycols, Chorinated or Sulphonated paraffines, mineral oil.	Baker et al. 1983
	Polyglycols, glycol ether, alcohol amine salts, little or no oil	Sutton et al., 1985
	Alkanolamine, emulsified oil.	Polak, 1986
	Sodium O, O-diethyl dithiophosphate, Methyldiethanolamine(MDEA)	Sherburn & Large, 1999
	2-amino-2ethyl-1,3,-propanethanol (AEPD), 2-(2-aminoethoxy) ethanol,	Geier et al. 2003
	Ethanolamine; 2-aminoethanol, N, N'-methylenebismorpholine	Castrol, 2002A
Semi-synthetic	Triethanolamine(1800-2100mg/L) sodium sulphonate (500-600mg/L), 2-ethoxyethanol (80-100mg/L)	Schreyer & Coughlin, 1999
	Alcohol ethoxylate phosphate ester, polysulphides, di-tert-deodecyl, alcohol, C11-14-iso, C13-rich, sodium sulphonate, 1-[2-(allyloxy)-2-(2,4-dichlorophenyl)]-1H-imidazole, N, N'-methylenebismorpholine	Castrol, 2002b
Not stated whether synthetic/semi-synthetic	Petroleum oil (1-5%), petroleum sulphonates (0.1-0.5%) linoleic acid (< 0.1%), oleic acid	Foxall-VanAken et al 1986
	Fatty acids, alkanolamines, alcohols, polyglycols, amino acids, carboxylic acids, surfactants containing sulphur, chloroalkanes, triazoles, triazines.	Kim et al., 1989; Kim et al., 1994
	Triethanolamine, cyclohexanamine, benzotriazole, indole, heptanoic acid	
	Decanoic acid, hexadecanoic acid, 9-octadecenoic acid.	Kim et al., 1994
	Mineral oil, sulphonated products, emulsifying agents	Deepak et al., 1994
	Alkanolamineborates, ethanolamines	
	Boric acid	Bruze et al., 1995
	Mineral oil (89%), nonyl phenol 10 MEO (3.5%), fatty acids (3.0%)	BLF, 2003
	Nonyl phenol 4 MEO (2.7%), ethoxylated alcohols (1.8%)	Portela et al., 2001
	Benzotriazole (16%), amine propoxylate (54%), propylene (8%), formaldehyde-based biocide,	
	benzotriazole, dodecanedioic acid, lauric acid, sebacic acid, amine propoxylate, glycerine, propylene glycol	Van der Gast et al. 2003a, b
Cleaners	Silici acid, dipotassium salt, ethanolamie; 2-aminoethanol, alcohol, C8-10 ethers with polyethylene polypropylene, glycol monobenzyl ether, sodium hydroxide, silicic acid, sodium salt, alcohol, C11-14-iso-C13-rich	Castrol, 2003

2.4 Characteristics of Metalworking Fluids

From the point of view of waste disposal, the complex formulations of MWFs and the various substances used in them are the major challenges in terms of the disposal of spent MWFs. The low biodegradability of mineral oils, biocides and alkaline reserves are normally highlighted in studies of MWF disposal, especially in those employing biological wastewater treatment methods in the disposal of spent MWFs. Meanwhile, there are many studies on developing alternative constituents for producing MWFs. For example, in some studies, vegetable oils were utilised as an alternative to petroleum/mineral oils, because they are environmental friendly, renewable, less toxic and readily biodegradable (Norrby, 2003; Matthew et al., 2007; Lawal et al., 2012).

For a study such as this one researching into biological degradation of MWFs, it is clear that the characteristics of MWFs must have a significant effect on the degradation route and the related microbes, including its toxicity and high pH, which together lead to low biodegradability. As has been introduced above, MWFs normally consist of mineral oil, water and various additives, and represent an extreme environment for the survival and growth of microorganisms (Perez et al., 2006). Despite this, some microorganisms do survive in MWFs where they can be significant problem in terms of premature bio-deterioration, as has been proved in many review studies (Cheng et al., 2005). Thus, it is important to consider the key characteristics of MWF that determine their degradability. The main ones considered to be important for the purposes of this study are: (1) high concentration of pollutants and inherent low biodegradability of MWFs; (2) the chemical characteristics of MWFs including pH and alkalinity; (3) the nutrient composition in

MWFs for growth of bacteria (C:N:P rate); and (4) the compounds of MWFs which have significant effects on the activity of microbes (bio-inhibitors including biocides and chlorinated hydrocarbon).

1) Biodegradability and Concentration

Low biodegradability is one of the major characteristics of MWFs; which means MWFs are quite difficult to be disposed via biological treatment processes. This characteristic helps MWFs maintain their functions for metal machining operations, but it also causes inhibitory effects on the activity and growth of bacteria in treatment systems. Theoretically, two key parameters are widely used to indicate the degradability and biodegradability of target pollutant/wastewater: Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD) (Metcalf & Eddy, 2004, pp. 81-98). The COD is the parameter used by industry and local authorities to measure the oxygen equivalent of the organic pollutant in wastewater that can be oxidised chemically using dichromate in an acid solution. The determination of BOD involves the measurement of the dissolved oxygen used by microorganism in the biochemical oxidation of organic matter. The ratio of BOD/COD is the typical value used to indicate the biodegradability of wastewater. If the BOD/COD ratio is 0.5 or greater, the wastewater is considered to be easily treatable by biological methods; however, if it is below 0.3, this indicates low biodegradability (Metcalf & Eddy, 2004, pp. 81-98). The literature review reveals that, in reported studies, most of the MWFs used as influents were simulated samples with quite high levels of COD concentrations; thus, they were all diluted to several thousand milligrams per litre in the experiments. In a study funded by the Department of Trade and Industry in the UK

(1998), the COD concentration of the MWF sample was shown to be 48,000-68,000mg/L. In Perez, et al.'s research (2006), the COD of the MWF sample was 2,500,000mg/L, and the ranges of the COD concentration of the MWFs used in Kim et al.'s study (1989, 1992, 1992a) were 51,000-768,000mg/L.

pH and alkalinity are amongst the two most important factors that determine the bio-degradation rate of MWFs. The literature review reveals that there is some confusion about the definitions of pH and alkalinity by some researchers such as Perez, et al. (2006). Basically, pH is defined as the negative logarithm of the hydrogen-ion concentration; it indicates the acidity or basicity of an aqueous solution. Alkalinity, on the other hand, is a parameter indicating the buffering capacity of an aqueous solution; it results from the presence of hydroxides [OH^-], carbonates [CO_3^{2-}], and bicarbonates [HCO_3^-] (Metcalf & Eddy, 2004, pp 31, 57, 58). Among the studies reviewed, the pH of MWF ranged from 8 to 11 with high alkalinity caused by alkaline reserve compounds. The high pH formulations of MWFs can enhance the activity of biocides and aim to inhibit the growth of microbes in MWFs (Rossmoore, 1981; Sandin et al., 1990; Cheng et al., 2005). The alkalinity values of the MWFs samples used in reported studies were not mentioned at all. This may be because most of the MWFs employed in industry contain alkaline reserve agents which can produce a high level of alkalinity in MWFs. In one study investigating the effect of pH value on the bio-degradation of spent MWFs, it was found that the optimal pH range for aerobic treatment of MWFs was between 6 and 7 (van der Gast et al., 2004).

2) Nutrient Composition

In regard to support bacterial growth, sources of carbon, nitrogen and phosphorus are crucial. From the composition of MWFs detailed above, it can be seen that one of the major constituents of MWFs are hydrocarbons refined from crude oil; these are an attractive carbon source for bacterial growth. Furthermore, it can also be seen that, MWFs also contain sufficient sources of nitrogen and sulphur. However, it is not clear whether MWFs have sufficient sources of phosphorus to support microbial growth. In previous studies, it has been demonstrated that adding a supplement of phosphorus could improve the growth of microorganisms, overall treatment efficiency and performance in the biological treatment processes of MWFs (Schreyer & Coughlin, 1999; Cheng et al., 2004).

3) Toxicity

The toxicity of MWFs can be defined as the degree to which MWFs' compounds can damage the microorganisms that come into contact with them or inhibit their growth and activity. More precisely, the toxicity of MWFs may include two aspects. The first is the presence of biocides, the key feature for controlling bacteria growth in MWFs. These biocides may be metallic, chlorinated organic compounds, formaldehyde-releasing compounds or organic sulphur nitrogen compounds (Rossmoore, 1981; 1983; Carvalhinha et al., 2010; Gilbert et al., 2010). Secondly, some degradation products from the substances in MWFs also have toxicity. Moreover, the high pH and low biodegradability causes some inhibitory effects on the growth of microbes.

The bio-deterioration of MWFs has been considered the most significant challenges when employing MWFs (Rossmoore, 1991). Bio-deterioration changes the chemical and physical characteristics of MWFs including their pH, viscosity and stability, and thus the quality of the MWF. These changes can cause the further degradation of MWFs. In consequence, the bio-deterioration of some MWFs may create problems when applying them to the machining of ferrous metals (Bakalova et al., 2007).

2.5 Disposal of Spent Metalworking Fluids

2.5.1 Overview of Industrial Wastewater Treatment

The activities of human society produce liquids, solid wastes and air emissions. Liquid waste is defined as wastewater—the water used from various applications. The sources generating wastewater can be categorised into two major types: domestic wastewater and industrial wastewater. Domestic wastewater, which is also called sewage, includes the used water discharged from residences, institutions and commercial establishments, and groundwater, surface water and storm water may also be present in domestic wastewater. Industrial wastewater (industrial effluents/nondomestic wastewater) is mainly generated from industrial sources such as factories and mills (Metcalf & Eddy, 2004, pp. 1-3). This type of wastewater normally requires particular treatment before it is discharged into the natural environment because it often contains a broad of contaminants.

Compared with domestic wastewater, industrial wastewater causes higher level of concerns regarding its health and environmental impact which has led to increasingly stringent policies and regulations. This is because industrial wastewater normally contains a variety of pollutants in high concentration, including inorganic pollutants

(inorganic salts, heavy metals, etc) and organic pollutants (hydrocarbon, nutrient). To date, many processes and technologies have been employed in the treatment of industrial wastewater including physical, chemical and biological processes. Generally speaking, there are three types of treatment approaches, physical, chemical and biological. In many cases, hybrid treatment processes are applied that combine complementary approaches (Metcalf & Eddy, 2004). The processes and techniques of the physical approach include screening, absorption, sedimentation, flocculation, flotation, and filtration (Rios et al., 1998; Skerlos et al., 2000; Hilal et al., 2004). The major chemical processes applied during wastewater treatment are coagulation, incineration and chemical oxidation including advanced oxidation processes, such as Fenton's oxidation, ozone oxidation and photocatalytic oxidation (Jagadevan et al., 2012; Yuan et al., 2011; Canizares et al., 2008). The biological treatment processes included in the biological approach to wastewater treatment can be classified into aerobic processes (such as conventional aerobic treatment processes using activated sludge) and anaerobic processes (such as the anaerobic activated sludge blanket system), or they can be categorised into suspended growth biological processes and attached growth biological processes (Buers et al., 1997; van der Gast et al., 2004; Muszynski & Lebkowska, 2005; Bakalova et al., 2007).

The applications and development of these treatment processes and technologies are driven by the relevant environmental policies and regulations.

2.5.2 Policies and Regulations of Industrial Wastewater Treatment

Environmental protection policies and regulations are made by governments in order to reduce the environmental impacts of human activities. Most of the regulations and

policies focus on pollution management, prevention and control rather than environmental recovery. In addition, some standards have been created for minimising the pollutants and contaminants being discharged into the natural environment. Some of the standards, regulations and policies on industrial wastewater management and treatment in the UK and EU are related to the disposal of spent MWFs.

One of the most important policies on water pollution prevention and control in the European Union in the last decade is the European Water Framework Directive. The purposes of the policy include: (1) to prevent deterioration and enhance the status of aquatic ecosystems and associated wetlands; (2) to promote sustainable water use; (3) to reduce pollution from priority substances; (4) to prevent deterioration of groundwater and (5) to contribute to mitigating the effects of floods/droughts (EC Water Framework Directive 2000). In the UK, the Water Framework Directive is transferred to local River Basin Management Plans (RBMPs) by the UK Environment Agency (UK Environment Agency, 2006). Another important policy which is relevant to industrial effluent treatment is the Integrated Pollution Prevention and Control (IPPC) Directive. The regulations involved in industrial effluent treatment include: the Environmental Permitting Regulations (EPR); the Water Act 2003; the Water Industry Act 1991; the Water Resources Act Regulations 2006, 2009, 2010; Control of Pollution Regulations 1996 & 2001; Environmental Damage Regulations 2009 (UK Environment Agency). Apart from the regulations on industrial effluent treatment, because MWFs contain some hazardous substances, there are some additional regulations which are related to the management and disposal of spent MWFs, such as the Oil Storage Regulations, Registration, Evaluation and Authorisation of Chemicals (REACH), Restriction of

Hazardous Substances (RoHS), Solvent Emissions Directive (SED), Waste Legislations, Hazardous Waste Regulations, Control of Major Accidents and Hazards Regulations 2005 (COMAH) and Control of Substances Hazardous to Health (COSHH). Most of the regulations listed above were amended recently, and have become tighter in regards to the disposal of industrial effluents and hazardous substances.

Due to tightening regulations with regards to toxic waste and the drive for more sustainable waste treatment methods, there is an increasing interest in the development of effective and sustainable disposal methods of spent MWFs.

2.5.3 Disposal Methods of Spent Metalworking Fluids

In general, the disposal methods in regard to spent MWFs can be classified into three categories, i.e. chemical, physical and biological methods (Cheng et al., 2005).

1. Chemical Treatment Methods

Chemical disposal methods can be defined as treatment methods which can change the chemistry or compositions of the substrates by dosing with inorganic or organic oxidising chemicals or employing chemical reactions. The chemical treatment methods applied in the disposal of spent MWFs include de-emulsification with chemicals, electro-coagulation and advanced oxidation processes (Rios et al., 1998; Cheng et al., 2005; Jagadevan et al., 2012).

In some previous studies, some inorganic and organic chemicals which can react with the emulsifiers in MWFs were used to break the emulsions into an aqueous phase and other fractions. The majority of the compounds in MWFs were separated via coagulation

occurring during the de-emulsification process (Rios et al., 1998; Yoshio & Masanori, 2001; Yuan et al., 2011; Cheng et al., 2005). However, de-emulsification with chemicals is just a method of separating the pollutants from the aqueous phase rather than decomposing them. Thus, some other methods, such as landfill and incineration, may have to be employed in the disposal of the concentrated solid pollutants which is an increasingly expensive route.

The electro-coagulation technique (EC) is also employed in the treatment of spent MWFs. It is an electrolysis process with reactive anodes made of iron or aluminium (Canizares et al., 2008). The main reactions at the anode and cathode electrodes are shown as follows (Fe electrode):

$$\text{Anode Reaction(s): } Fe \rightarrow Fe^{3+} + 3e^{-} \qquad Fe \rightarrow Fe^{2+} + 2e^{-}$$

$$\text{Cathode Reaction: } 3H_2O + 3e^{-} \rightarrow \frac{3}{2}H_2 \uparrow + 3OH^{-}$$

During the EC process, hydrolysis products are formed and interact with the organic molecules in wastewater. The hydrolysis products have amorphous structures with very large surface areas and positive charges, which causes precipitation and adsorption. A few studies have reported on the treatment of synthetic and semi-synthetic MWFs using the EC process; however, EC is not popularly applied in wastewater treatment because of its high cost in terms of capital investment and energy consumption (Muszynski et al., 2009; Kobya et al., 2008; Canizares et al., 2008).

Advanced oxidation processes (AOPs) are commonly used to oxidise complex organic constituents in wastewater which cause difficulties during biological treatment processes, such as the petroleum compounds in MWFs (Jagadevan et al., 2012). AOPs are oxidation

processes using hydroxyl free radical (HO*), which is one of the strongest oxidants used to decompose compounds that cannot be oxidised by oxidants such as oxygen (Metcalf & Eddy, 2004). Based on the studies reviewed (Metcalf & Eddy, 2004, p. 1197; Munter, 2001), the oxidising power of various oxidants is summarised in Table 2.2.

Table 2.2: Oxidising potential of some oxidising agents

Oxidising Agent	Electrochemical Oxidation Potential (EOP), V
Fluorine	**3.06**
Hydroxyl Radical	**2.80**
Ozone	**2.08**
Hydrogen Peroxide	**1.78**
Hypochlorite	**1.49**
Chlorine	**1.36**
Chlorine Dioxide	**1.27**
Oxygen (molecular)	**1.23**

(Adapted from Metcalf & Eddy, 2004, p1197; Munter, 2001)

There are several technologies used to produce HO* in the aqueous phase including Ozone/UV, Ozone/Hydrogen Peroxide, Hydrogen Peroxide/UV, Fenton's reaction etc. Most of the AOPs are currently under development (Glaze et al., 1987; Munter, 2001; Alaton et al., 2009; Gogate & Pandit, 2004; Gerrity & Snyder, 2011; Joseph, et al., 2009, Jagadevan et al., 2012). In addition, some hybrid treatment processes coupling AOPs and biological treatment processes were employed in previous studies. For example, Jagadevan et al. (2012) developed a hybrid nano-zerovalent iron initiated oxidation with aerobic wastewater treatment process in the disposal of recalcitrant waste MWFs.

2. Physical Treatment Methods

Physical treatment methods employed in the disposal of MWFs mainly include evaporation, membrane filtration, microfiltration, ultrafiltration, nanofiltration, reverse osmosis (RO) and peat adsorption (Rios et al., 1998; Skerlos et al., 2000; Hilal et al., 2004; Cheng et al., 2005). These physical disposal methods are based on the separation of the pollutants in wastewater from the aqueous phase and treating each phase independently. However, due to the tightening of regulations on air emissions, evaporation is now forbidden from being employed in the disposal of hazardous wastes, including MWFs, in many developed countries (Cheryan &Rajagopalan, 1998).

With the rapid development of filtration technologies in the last decade, filtration processes, including membrane filtration, microfiltration, ultrafiltration, nanofiltration, and reverse osmosis (RO), have been widely employed in wastewater treatment, as well as in potable water treatment. In general, filtration processes can be catalogued by the pore dimension (Cheng, et al., 2005; Skerlos, et al., 2000). Compared with other treatment methods, membrane filtration technologies have several advantages in the treatment of spent MWFs (Chang et al., 2001; Hilal, et al., 2004), including:

- Membrane filtration can be employed in treating a large variety of spent MWFs with relatively high performance and removal efficiency of pollutants.
- No extraneous chemicals are needed.
- Membrane filtration technologies can be used to recycle some selected substances in spent MWFs.
- Effluent treated using nanofiltration (pore sizes range from 1 to 10 angstrom) and

ultrafiltration (pore sizes range from 0.01 to 0.1 micron) can be considered microorganism-free.

- Membrane equipment has low footprint and energy costs.
- The membrane filtration plant can be automated and requires highly skilled operators.

However, there are two hurdles to employing membrane filtration processes in the disposal of spent MWFs, including concentration polarisation and membrane fouling (Hesampour, et al. 2008). The literature review reveals that, in order to overcome these short-comings, there have been some studies investigating the effects of operational factors, including transmembrane pressure, flux, feed velocity, feed concentration, pH and the type of membrane process on these pinch-points (Benito, et al., 2000; 2001; Balkacem, et al., 1995; Kabsch-Korbutowicz, 2006; Ezzati et al., 2005).

3. Biological Treatment Methods

There is a relatively long history of employing microorganisms in the treatment of wastewater. Microorganisms can oxidise the organic pollutants in wastewater into simple end products. The conventional biological wastewater treatment process was developed around the beginning of the 18th century, and was employed to treat domestic wastewater. The first successful biological sewage water treatment plant was built in Manchester in the UK between 1910 and 1914 (Scragg, 1999, p. 46). Compared with chemical and physical treatment methods, biological treatment has several advantages, such as the fact that it is relatively cost-effective, no chemicals are needed and it is more sustainable for large scale wastewater treatment works. To date, biological treatment methods have been widely employed in industrial effluents treatment including aerobic and anaerobic

treatment processes. Aerobic and anaerobic treatment processes are categorised by the type of microorganism involved in the treatment processes. Buers et al. (1997) and Bakalova et al. (2007) reported that the components of MWFs can support the growth of selected bacteria as sole sources of carbon and nitrogen. However, MWFs also contain biocides, and this may cause inhibitory effect on the microorganisms in biological treatment processes and, ultimately, the failure of the entire operation. Schreyer and Coughlin (1999) and Cheng et al. (2004) reported that dosing using additional phosphorus enhances treatment performance and efficiency.

There are few reports of aerobic wastewater treatment processes with aerobic activated sludge being employed in the disposal of MWFs; the aerobic activated sludge processes may not be suitable for the disposal of highly concentrated industrial effluents because of the high energy consumption and production of a large amount of biomass (Backer et al., 1983; Cheng et al. 2005). However, there have been several studies on the aerobic treatment of MWFs with isolated aerobic bacteria and membrane bioreactors (Buers et al., 1997; van der Gast et al., 2004; Muszynski & Lebkowska, 2005; Connolly et al., 2006 & Bakalova et al., 2007). In these studies, the researchers isolated the aerobic bacteria from waste MWFs and used the isolates to treat their MWF samples. Some of them reported relatively good removal efficiencies (60—90%) of COD and BOD. The immobilisation of the isolates in the treatment units was also considered in some studies, such as using a PVC foam carrier (Muszynski & Lebkowska, 2005). In some other studies, the aerobic treatment processes were applied with a membrane bioreactor as the primary treatment process, and their results demonstrated good removal efficiency in regard to the COD and nitrogen of simulated semi-synthetic MWFs (Knoblock et al., 1994, Schuch et al., 2000,

Anderson et al., 2009). However, most of the studies were carried out with limited experimental conditions (such as low concentration of MWF samples and additional supplement of nutrients) and with no characterisation of MWFs samples (unknown composition of the MWF samples).

The anaerobic treatment of industrial wastewater has been a viable method in recent decade with the development of high-loading rate reactors, such as the anaerobic filter reactor and sludge blanket reactors (Fang, et al., 1996; Dinsdale, et al., 1997; Lettinga, 1996; Shink, 2002).

Compared with the aerobic wastewater treatment approach, the anaerobic treatment approach has a number of significant advantages (Fang, et al., 1996; Shink, 2002), including:

- High concentration of biomass, but no large volume of biomass generated so no biomass disposal required.
- Low energy cost and carbon emission
- No need to be fed continuously
- Less space required and controlled odours.
- Energy recovery from biogas generation in the anaerobic degradation process of organic pollutants.

On the other hand, it also has several disadvantages compared with the aerobic approach, including (Fang, et al., 1996; Shink, 2002; Cheng, et al., 2005):

- Anaerobic microorganisms are more sensitive to toxic matters, especially methanogens.

- The growth rates of anaerobic microorganisms are quite low.

Anaerobic treatment processes were also employed in the disposal of spent MWFs. The literature review reveals that this was a really challenging undertaking. Only a few studies were reported in this field in last decade. Further details regarding anaerobic degradation and treatment of MWFs will be given in section 2.7.

2.6 Microbiology of Waste Metalworking Fluids

The microbiology of spent MWFs is a significant issue in this study in terms of employing biological treatment methods in the disposal of MWFs. A variety of microbial communities may exist in MWFs, and their existence has a significant influence on the quality, performance and disposal of MWFs. On one hand, the colonisation and growth of bacteria in MWFs lead to some usage issues of MWFs in terms of performance, health and safety, such as deterioration and epidemiology (Hill, 1983; Jarvholm, 1981; Zeman et al., 1995; Moore et al., 2000). On the other hand, the economical and sustainable disposal of spent MWFs depends on the studies into the microbes; they have been successfully employed in the disposal of MWFs in some cases (van der Gast, et al., 2001; Khan & Yadav, 2004; Rhodes et al., 2008).

A range of technologies have been employed in studies to investigate the microbiology of MWFs. In some studies, only traditional bacteria isolation and microscope observation approaches were used to identify the anaerobic bacteria colonising in MWFs; however, the efficiency of this approach was quite low, in some cases, less than 1 % of the cells present were reported to have been isolated (Amann et al., 1995; Cheng et al., 2005; Saha

& Donofrio, 2012). With the development of molecular biology, some analytical techniques relating to a bacteria's genetic fingerprint have been invented and employed in the microbiological investigations, such as Fluorescence *In-Situ* Hybridisation probe (FISH) and quantitative real-time polymerase chain reaction (PCR) (Saha et al., 2012; van der Gast et al., 2001; Khan & Yadav, 2004; Rhodes et al., 2008). These techniques involve the binding of complementary oligo probes to a target sequence in the host ssDNA. Real-time PCR is a sensitive technique with cultivation-independent detection and quantification, which means that sample cultivation is not required for environmental microbial samples (Scherr et al., 2012; Green & Sambrook, 2012, pp. 455-681). Molecular-based techniques can be used not only to identify individual bacteria but also microbial communities. One of the most common and widely-used techniques for analysing microbial communities is Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis (PCR-DGGE); other techniques newly described in recent studies include 454 prosequencing and Illumina (Solexa) sequencing (van der Gast, et al., 2001; Mikkelson et al. 2009; Dey & Brenner, 2011).

A wide variety of microorganisms have been known to inhabit MWFs as the components of MWFs can support the growth of microorganisms including some pathogens. According to the studies reviewed, the microorganisms indentified in MWFs include *Staphylococcus*, *Streptococcus*, *Alcaligenes*, *Acinetobacter*, *Mycobacterium*, *Tessaracoccus lubricantis*, *Penicillium*, *Pseudomonads, Aspergillus*, *Fusarium*, *Cladosporium*, and sulphate-reducing bacteria; it was also reported that some of the microorganisms are commonly isolated from spent and in-use MWFs as dominant groups, such as *Pseudomonads* (van der Gast, et al., 2001;Mattsby-Blatzer et al., 1989; Perkins &

Angenent, 2010; Sandin et al., 1991; Moore et al., 2000; Gilbert et al., 2010; Rhodes et al., 2011; Saha & Donofrio, 2012).. The pathogens reported in MWFs include *Legionella sp*., *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Escherichia coli* (Bakalova et al. 2007; Cheng et al., 2005; Elsmore, 2003; Lucchesi et al., 2012; Saha & Donofrio, 2012). Fungi, such as yeast and mould, were also found in MWFs (Liu et al., 2010; Cheng et al., 2005, van der Gast, et al., 2001, 2003b). The isolated frequency of the 66 aerobic bacteria isolated from spent MWFs in van der Gast et al's study (2001) is shown in Figure 2.1.

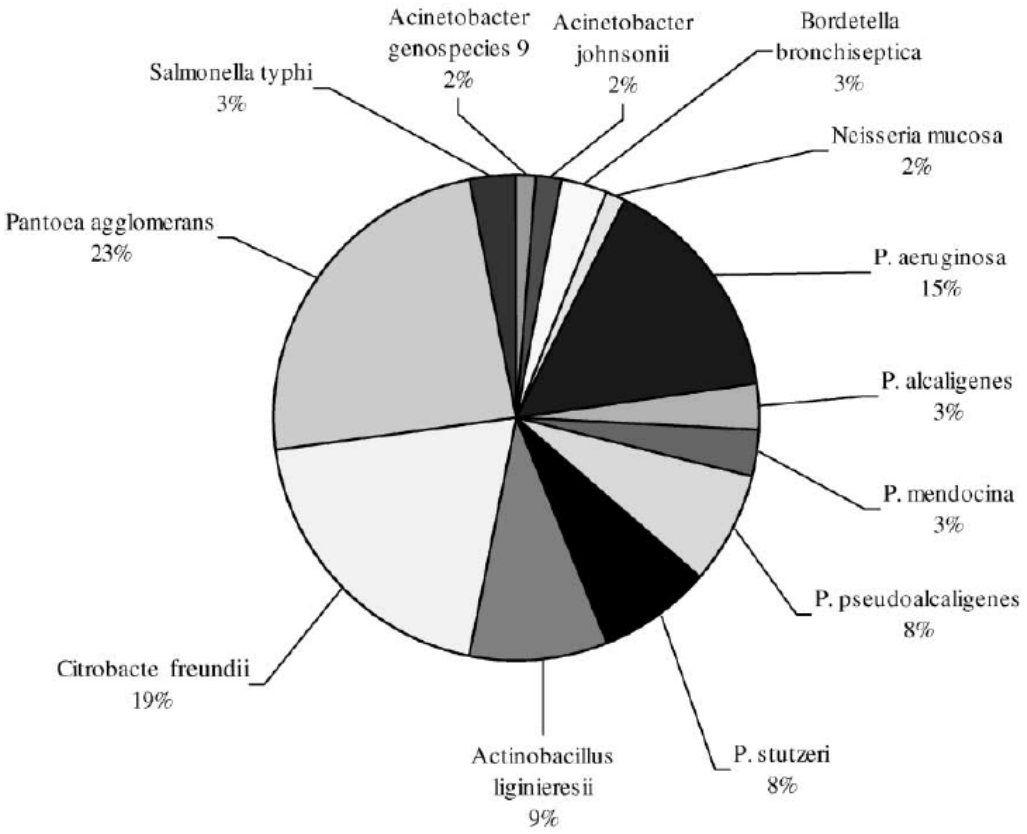

Figure 2. 1: Isolation frequency of the 66 bacterial isolates reported in van der Gast et al's study (2001).

The microbial contamination caused by the colonisation of microbes can change the stability, quality and performance of MWFs, but the exact roles of microorganisms in the

bio-degradation process of MWFs are as yet unknown. On the one hand, the existence of the microorganisms explains the deterioration of MWFs. On the other, it also shows that employing the microorganisms indigenous to MWFs has the potential to enhance the treatment efficiency of traditional biological methods in the disposal of spent MWFs since the population are acclimated to the extreme conditions. Baker et al. (1983) investigated the bacteria in activated sludge used for the treatment of MWFs. Four species were reported in Baker et al.'s study: *Acinetobacter, Alcaligenes, Flavobaterium* and *Pseudomonas*. It should be noted that *Acinetobacter* is one of the natural degraders of hydrocarbons in soil. van der Gast et al. (2001; 2003a, b; 2004) reported that an aerobic bacterial consortium was successfully applied to treat MWFs.

2.7 Anaerobic Degradation and Treatment of Metalworking Fluids

2.7.1 Overview of Anaerobic Degradation Mechanism

Anaerobic degradation, which is referred to as anaerobic digestion in some studies, is defined as a series of processes in which microorganisms decompose biodegradable material, in the absence of oxygen, into carbonic acids, alcohols, CH_4 and CO_2 (Scragg, 1999). There are several kinds of microorganisms, involved in the process of anaerobic degradation, such as hydrolytic, acid-forming bacteria (acidogentic & acetogenic bacteria) and methanogens; these form a complex micro-ecosystem. In the 1980s, Bryant (1979) proposed a theory in which the anaerobic degradation process was divided into three stages and the function of hydrogen producing acetogenic bacteria in anaerobic microbial system was highlighted. In Bryant's theory, the steps of anaerobic degradation process include hydrolysis and acidogenesis, acetogenesis, methanogenesis, as shown in Figure

2.2. Each step is performed by a specific type of anaerobic microorganisms. It shows that the anaerobic degradation process includes various biological conversion processes producing intermediate metabolite products. Dhaked et al., (2010) suggested that a sufficient anaerobic degradation process is at a balance status so as to avoid the accumulation of any intermediate metabolite in the system.

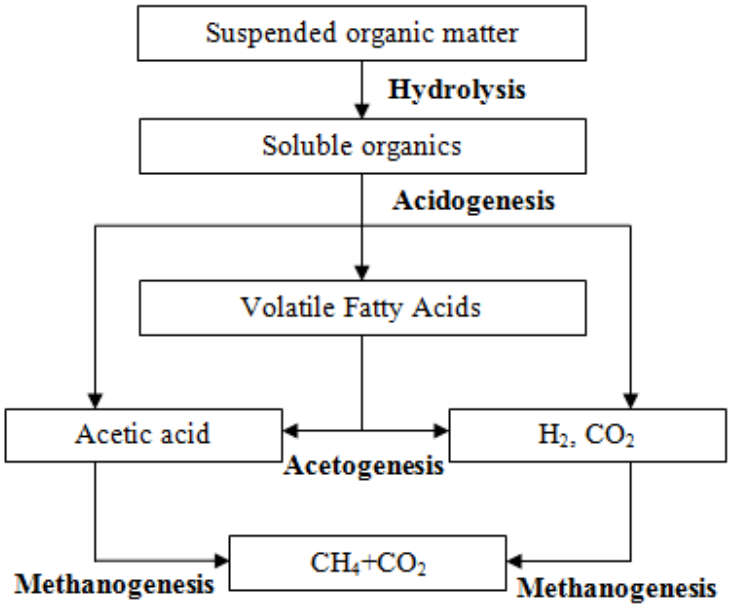

Figure 2.2: Steps in the anaerobic degradation process (Adapted from Appels et al., 2008)

The anaerobic degradation process of glucose ($C_6H_{12}O_6$) is given as an example as follows:

(1) Hydrolysis and Acidogenesis

$C_6H_{12}O_6 \rightarrow CH_3CH_2COOH+CH_3COOH+CO_2+H_2$

$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH+2CO_2+2H_2$

$CH_3CH_2CH_2COO^-+2H_2O \rightarrow 2CH_3COO^-+H^++2H_2$

(2) Acetogenesis

$CH_3CH_2COO^-+3H_2O \rightarrow CH_3COO^-+H^++HCO_3^-+3H_2$

$CH_3CH_2OH+H_2O \rightarrow CH_3COO^-+H^++HCO_3^-+2H_2$

$CH_3CHOHCOO^-+2H_2O \rightarrow CH_3COO^-+H^++HCO_3^-+2H_2$

(3) Methanogenesis

$HCO_3^-+H^++4H_2 \rightarrow CH_4+3H_2O$

$CH_3COO^-+H_2O \rightarrow CH_4+HCO_3^-$

$2CH_3CHOHCOO^-+SO_4^{2-} \rightarrow 2CH_3COO^-+2HCO_3^-+2H^++S^{2-}$

Glucose is a hydrocarbon compound with a simple molecular structure and its anaerobic degradation is a simple degradation process for anaerobic bacteria. As can be seen from the chemistry of MWFs, the majority of MWFs' ingredients are hydrocarbons with high molecular weights and complex molecular structures.

2.7.2 Anaerobic Degradation Metabolism of Hydrocarbons

The majority of the compositions of MWFs are petroleum products which consist of many different types of hydrocarbons. In general, hydrocarbons can be classified into: (1) saturated compounds (aliphatic and alicyclic alkanes); (2) C-C double bonds compounds (alkanes); (3) C-C triple bonds compounds (alkynes); and (4) monocyclic, polycyclic aromatic hydrocarbons (Pine, 1987; March, 1992). In the reported studies, it has been demonstrated that hydrocarbons which are anaerobic biodegradable include: linear, branched and cyclic alkanes (C6-C20), alkenes, monocyclic alkylbenzenes, benzene and naphthalene (Heider et al., 1999; Dolfing et al., 1990; Rueter et al., 1994; Rabus &

Widdel, 1995; Harms et al., 1998; Scherr et al., 2012). Some hydrocarbons can be degraded by pure bacterial cultures under anaerobic conditions (Heider et al., 1999). For example, it has been reported that many strains can degrade toluene anaerobically, such as Strain ToN1, Strain T3, Thauera aromatic K172, etc (Anders et al., 1995; Rabus & Widdel, 1995; Hess et al., 1997). Strain Hxd3, Pnd3 and TD3 are reported be capable of decomposing alkanes (C6-C20) under anaerobic conditions (Aeckersberg et al., 1991; Aeckersberg et al., 1998; Rueter et al., 1994). In addition, it has also been highlighted in some studies that the anaerobic degradation processes of hydrocarbons involve some different terminal electron acceptors including nitrate, sulphate and ferric iron (Siegert et al., 2011; Hasinger et al., 2012; Scherr et al. 2012).

2.7.3 Biomethanation and Methanogens

In the process of anaerobic degradation, the biomethanation process is significant in converting organic matter into methane. As was introduced above, methanogens can use the metabolites of other anaerobic bacteria (acetate, H_2) to generate methane. This study aims to reduce the inhibitory effect of the MWFs and enhance the methane production in the degradation process of MWFs. However, there are some big challenges involved in such a process.

Firstly, it is extremely hard to isolate and cultivate methanogens. According to the methanogen community's analysis of some of the latest reported studies, most of the methanogens in the anaerobic digestion systems are individually unculturable and can only be isolated in culture as a consortium (Scherr et al., 2012; Godin et al., 2012; Kim et al., 2012; Sheppard et al., 2005; Singh et al., 2012). Methanogens are obligate anaerobic

bacteria; theoretically, they cannot survive in the presence of oxygen, which also causes difficulties in terms of the isolation and manipulation. Additionally, methanogens are very sensitive to changes of temperature and pH. These changes may affect the activity of methanogens, the community structure and the degradation pathways of organic matter. It was pointed out that that the Gibbs free energy, ΔG, of methanogenesis is much lower than that of the nitrate reduction pathway and sulphate reduction pathway (Heider et al. 1999; Widdel & Rabus, 2001). A low pH growth condition can reduce the activity and growth of methanogens (Zoetemeyer et al., 1982; Visser et al., 1993; Anderson & Yang, 1992). All of this makes it extremely hard to isolate pure strains of methanogens.

2.7.4 Anaerobic Treatment of Metalworking Fluids

The literature review reveals that, in previous studies, the anaerobic treatment of MWFs has received very little attention. One comprehensive study on the anaerobic treatment of MWFs was carried out by Byung R. Kim et al. (1989; 1990; 1992). They carried out a series of studies with a simulated MWF; the treatment schemes carried out were anaerobic, anaerobic followed by aerobic and straight aerobic. In the reported studies, the potential of anaerobic COD, organic nitrogen and fatty acids removal in the three treatment schemes was investigated by employing a granular activated carbon (GAC) fluidised-bed reactor (Kim et al., 1989; 1990; 1992a). The conclusions of their study on anaerobic COD removal include:

(1) Biodegradable organics were efficiently degraded at relatively high COD removal rates (more than 70kg/m^3·d);

(2) Approximately 35% of the influent organics was found to be anaerobically non-biodegradable;

(3) Approximately 68% of the biodegraded COD was converted to methane and the rest went to biomass and dissolved gases;

(4) The yield coefficient of this anaerobic process was estimated to be approximately 0.09 g biomass produced /g COD degraded;

(5) The overall steady-state removal rate of biodegradable COD could be described by first-order kinetics;

(6) Influent COD concentration up to 5,324mg/L did not appear to be toxic to microorganisms.

In Kim et al.'s study (1994) on the biological removal of organic nitrogen and fatty acids, two major organic groups in synthetic MWFs — i.e. nitrogen-containing organics (primarily alkanolamines and heterocyclic compounds) and fatty acids — were investigated in regard to their biodegradability in three biological treatment schemes, including anaerobic, anaerobic-aerobic and straight aerobic treatment schemes. In general, it was concluded that the organics were well degraded (more than 90% COD removed) through either anaerobic-aerobic or straight aerobic treatment while the identified fatty acids were found to be well degraded both anaerobically and aerobically.

In summary, Kim et al.'s studies show great potential for the treatment of MWFs using anaerobic treatment processes and anaerobic-aerobic hybrid treatment scheme. However, because of the unknown composition of their MWF and the adsorption effect of GAC,

the mechanism of COD removal and effects from MWFs on the microbial communities were not investigated or discussed.

Another study on the anaerobic treatment of MWFs was carried out by Perez et al. (2006) in an up-flow anaerobic fixed-film reactor (UAFF). In their study, the effect of co-substrate on the thermophilic anaerobic degradation of an unknown MWF was investigated, but the methodology of this study was defective and the conclusions are not clear. For example, the authors still had no idea if the COD of the MWF was removed biologically or physically after 130 days of operation.

To date, apart from Kim et al.'s and Perez, et al.'s studies, there is nearly no systematic studies in the anaerobic treatment of MWFs published in the fields of academic research or engineering; and there is a real need for some quality studies.

2.7.5 Environment Affecting Factors of Anaerobic Treatment Processes

According to the published studies on the microbiological system of anaerobic degradation, there are a variety of microbial functional groups involved in anaerobic degradation such as methanogens, acidogens and hydrolytic bacteria. (Lettinga et al., 1980). Many factors can affect the activity of bacteria and the results of system operations (Show et al., 2004; Hulshoff Pol et al., 2004). These affecting factors can be classified into four major types: the characteristics of wastewater (chemical constituents of wastewater, concentration and their toxicity), seeding inocula (selection of inocula, quantity and activity), environmental conditions (temperature, pH, and alkalinity, balance of nutrients) and operational conditions (reactor design, loading rates, start-up procedures,

liquid and gas up-flow velocities, hydraulic retention time and mixture) (Show et al., 2004; Ghangrekar et al., 2005; Hulshoff Pol et al., 2004; Sun et al., 2000; Gonzãlez et al., 1998). In this study, the factors which are directly related to the composition and characteristic of a MWF are the key concern to be discussed. These factors include: (1) the COD, pH and alkalinity of the MWF; (2) the balance of nutrients of the MWF; (3) substances in the MWF; (4) the concentrations of the substances; and (5) their toxicity to the microorganisms employed in the degradation system.

The COD concentration of a MWF is one of the most important factors affecting the degradation of the MWF, which was highlighted and discussed in almost every reported study. Normally, high COD concentration is accompanied by a high concentration of toxic background in MWFs. According to Kim et al.'s study (1992a), a COD concentration up to 5,324mg/L has no significant toxicity on microorganisms in the anaerobic degradation process. However, there was no standardised toxicity analysis method developed in Kim's studies or other similar studies.

pH is a key determining factor in anaerobic degradation and the efficient operation of anaerobic reactors (Anderson & Yang, 1992). It has direct effects on the methane yield of anaerobic degradation systems, with the optimal range of the pH value for methanogenic activity being between 6.3 and 7.8 (van Haandel & Lettinga, 1994). Nonetheless, the pH values of MWFs are normally quite high because of the alkaline reserve agents (normally pH > 8).

The nutrient balance of wastewater/MWFs is expressed as C: N: P or COD: N: P. It has been reported that the best C: N: P ratio to anaerobic degradation is in the region of

75:5:1, the COD: N: P about 200:5:1. Some previous studies reported that MWFs may contain sufficient sources of carbon, nitrogen and sulphur (Schreyer & Coughlin, 1999; Cheng et al., 2004), but this cannot be certain in regard to every type of MWF waste stream or in deed information. In some cases, nitrogenous fertiliser, livestock excrement and other materials which contain nitrogen in high concentrations can be used to amend the nitrogen compositions to stimulate greater microbial activity (Sun et al., 2000).

In addition, selecting the experimental temperature is also important. It has been reported that, temperature has significant effects on the activity, diversity and community structure of anaerobic microorganisms, especially on methanogens (Boone, D. R., 1987; Kim et al., 2004; Lone, et al., 2008). Among the previous studies in anaerobic degradation and reactor operation, three experimental temperature conditions were investigated: an ambient range (18℃～30℃), a mesophilic range (30℃～40℃) and a thermophilic range (50℃～60℃). In general, the bacterial activity and loading rates of reactors operating at lower temperatures were less than those at normal and higher temperatures (El-Mashad et al., 2004; Xie & Liu, 2007). Considering the energy cost and degradation efficiency of the anaerobic ecosystems operating at these three temperature ranges in reported studies, mesophilic anaerobic technology is more favoured because of its relatively high treatment efficiency and low energy cost. On this basis, this approach was selected for the current study.

2.7.6 Inhibitors of Anaerobic Degradation

As introduced above, unfavourable environmental factors may cause inhibitory effect on the anaerobic degradation process. Besides these factors, there are some other inhibitors

of the anaerobic degradation process. Some of them have been mentioned in previous sections (such as biocides), but they are systematically summarised and highlighted in this section, and include ammonia, sulphide, heavy metals, the presence of some ions and organics (Chen et al., 2007).

Ammonia is normally produced by the bio-degradation of nitrogenous matters, and ammonia inhibition effects microorganisms through several mechanisms, such as changing intracellular pH, inhibiting specific enzyme reaction and causing proton imbalances (Kayhanian, 1999; Whittmann et al., 1995; Gallert et al., 1998). It has been reported that, in the anaerobic digestion system, methanogens are relatively more sensitive to ammonia inhibition compared with other types of anaerobic bacteria (Kayhanian, 1994; Koster & Lettinga, 1984; Zeeman, 1985; Jarrell et al., 1987; Chen et al., 2007).

Sulphate is commonly contained in many types of wastewater, including MWFs. In the anaerobic digestion system, sulphate is converted to sulphide by sulphate-reducing bacteria (SRB), and the toxicity of sulphide can inhibit some types of anaerobic bacteria such as methanogens. Besides the toxicity issue, the competition between SRB and methanogens for common organic and inorganic substrates is another reason for inhibitory effect (Hilton & Oleszkiewicz, 1988; Harada et al., 1994; Anderson et al., 1982; Colleran et al., 1998).

Heavy metals may be present in significant concentration in MWFs, particularly if the fluid has been used to work particular metals. Heavy metals include cobalt, copper, zinc, chromium, cadmium and nickel; which are biologically very toxic. It has been reported

that the toxicity of heavy metals is one of the major causes of anaerobic digester inhibition or failure (Chen et al., 2007). Heavy metals are not biodegradable and can be accumulated in microorganisms to toxic concentration, while also causing disruption of enzyme function and structure in microbes, which is also observed as toxicity in previous studies (Vallee & Ulner, 1972; Mori et al., 2000). The presence of some light metal ions, such as Mg^{2+}, Ca^{2+} and Al^{3+}, may also entail salt toxicity to the anaerobic digestion system. Light metal ions are known to be essential for the growth of microorganisms, but excessive amounts of these ions can cause inhibition or toxicity (Soto et al., 1993; Chen et al., 2007).

Apart from inhibitions caused by inorganic matter, a wide range of organic compounds have been reported to be toxic to anaerobic degradation processes. These organic compounds include alkyl benzenes, halogenated benzenes, phenol and alkyl phenol, halogenated phenols, nitrophenols, halogenated aliphatics etc (Chen et al., 2007). Moreover, some surfactants and detergents were also reported to adversely impact anaerobic degradation processes (Gavala & Ahring, 2002; Madsen & Rasmussen, 1996). Water-soluble MWFs normally contain various hydrocarbons, surfactants and detergents.

2.8 Research Aim and Objectives

The overall aim of this study is to employ the anaerobic degradation process in the disposal of MWFs and recover energy from the wastes by methangenesis. The major reason for the selection of the anaerobic degradation process rather than the aerobic degradation process is: the anaerobic degradation process can produce methane as a

source of energy recovery; on the contrary, a large amount of CO_2 emissions are produced in the aerobic degradation process of MWFs.

The major challenge of this study was to biologically reduce the inhibitory effects of the MWF on the anaerobic microorganisms involved in the degradation process, especially on methanogens, functional group which is very sensitive to extreme growth environments and toxic matter. The inhibitory effect is often referred to as toxicity.

To achieve the aim mentioned above, the major objectives of this study include:

I. To investigate the anaerobic degradation of the MWF and its inhibitory effects on the degradation process;
II. To investigate the inhibitory effects of the MWF on methanogenesis; and
III. To overcome the toxic effects of the MWF on anaerobic degradation process employing anaerobic bacterial cultures isolated from spent MWFs.

Chapter 3

Anaerobic Biodegradation of Metalworking Fluids

3.1 Introduction

Metalworking fluids (MWFs), which contain highly concentrated petroleum components and various additives with complex formulations, have been widely employed in modern industries and play a central role in machining processes. With the development of MWFs, more and more compounds have been used in their formulations (Byers et al., 1994; Rabenstein et al., 2009). The worldwide annual usage of MWFs now exceeds two billion litres, and the spent MWFs have become a major environmental issue as a consequence (Cheng et al., 2005). Due to ever tightening policies and regulations on water and environment protection, there is a huge emerging demand for efficient and sustainable disposal methods for spent MWFs.

To date, chemical, physical and biological wastewater treatment methods have been employed in the disposal of MWFs, with great interest in biological wastewater treatment methods, as they are more flexible, less costly than the other methods and most importantly sustainable. In particular, the anaerobic degradation route can break down organic pollutants and convert them to biogas (Rios et al., 1998; Metcalf & Eddy, 2004; Hilal et al., 2004; Cheng et al., 2005). Compared with the aerobic degradation route, the

anaerobic degradation route requires much less energy consumption. It also removes sulphate and produces exploitable biogas instead of the CO_2 emissions generated in the aerobic degradation route. The literature review reveals that the anaerobic treatment method, in comparison to other methods, has received very little attention in regard to the disposal of MWFs. This may be because of the two challenges of employing anaerobic degradation in the disposal of MWFs. Firstly, anaerobic microorganisms, especially methanogens, have specific growth requirements and are very sensitive to high pH, toxic components, and petroleum compounds (Kim et al., 1989; Cheng et al., 2005). Secondly, a wide variety of chemicals and complex formulations, which are seldom divulged by manufacturers because of their commercial sensitivity, are employed in MWFs. These make biological treatment particularly challenging.

Several studies have found that the compounds in MWFs had inhibitory effects on the anaerobic degradation process, especially on methanogenesis; however, there is still insufficient information and data regarding the inhibitory effects (Kim et al., 1989; 1992; 1992a; Perez et al., 2006). Lack of information on the formulation of the MWFs used in the reported studies has made it extremely difficult to estimate and understand the anaerobic biodegradability of the MWF samples employed in the studies and the inhibitory effects on anaerobic degradation process. The BOD/COD ratio is typically employed to indicate the biodegradability of wastewater (Metcalf & Eddy, 2004, p 97), but it is not suitable for highly concentrated organic wastewater containing biocides such as MWFs. This is because the BOD value is measured using aerobic bacteria and their activity may be significantly inhibited by the presence of toxic components, such as biocides, added specifically to make the substrate unpalatable.

The studies presented in this chapter focus on investigating the anaerobic degradation of the MWF in experimental systems inoculated with flocculent and granular anaerobic sludge.

A typical semi-synthetic MWF was selected for this study as it contains both mineral oil and additives. The study described in this chapter was an investigation the anaerobic degradation of the MWF, and it consisted of three major sections:

1）An investigation of the key characteristics of the MWF, particularly of parameters likely affect the operation of further experiments, such as sample concentration and pH value.

2）An investigation of the anaerobic degradation of the MWF in experimental system inoculated with anaerobic activated sludge.

3）An assessment of the potential inhibitory effect of the MWF on the anaerobic microorganisms in the experimental systems.

3.2 Materials and Methods

3.2.1 Metalworking Fluid Sample

The MWF investigated in this study was RELUBRO 800 BIO (MacDermind plc, Birmingham, UK), a typical semi-synthetic MWFs. It is a high performance chlorine-free MWF and can be employed as a lubricant in metal machining for a diverse range of applications. RELUBRO emulsion shows excellent resistance to bacteria and fungi colonisation and it represents the latest development in hazard label-free MWFs. Its formulation details are only partially divulged by the manufacturer for the aforementioned commercial reasons, but its major ingredients are provided in its safety data sheet, as shown in Table 3.1.

Table 3.2: Information on ingredients of RELUBRO 800 BIO

Name	EC No.	CAS-No.	Content
Hydrotreated light napthenic distillate	265-156-6	64742-53-6	30-60%
2-(2-Butoxyethoxy) Ethanol	203-961-6	112-34-5	5-10%
Benzenesulphonic acid (di-C10-18 alkyl derivatives, sodium salts)	298-640-0	93820-59-8	1-5%
1, 6 Dohydroxy-2, 5-Dioxohexane	222-720-6	3586-55-8	1-5%
2-Pyridinthiol-1-Oxide Na salt	223-296-5	3811-73-2	< 1%

(Adapted from safety data sheet of RELUBRO 800 BIO)

The sample of RELUBRO MWF used in this study was collected from the mixture tank of a metal-cutting machine in the workshop of the University of Oxford's Physics Department, and kept in a fridge in order to minimise its bio-deterioration.

According to the analytical methods described in section 3.2.5, several major parameters of the MWF sample were determined, and the results are shown in Table 3.2 below.

Table 3.3: Characteristics of the in-use MWF sample

Parameters	Mean Value
Chemical Oxygen Demand (COD)	100,000mg/L
Biochemical Oxygen Demand (BOD)	45,000mg/L
BOD/COD ratio	0.34
Total Organic Carbon (TOC)	62,500mg/L
Total Nitrogen (TN)	1,940mg/L
Nitrate	119mg/L
Sulphate	20,325mg/L
pH value	8.72

3.2.2 Anaerobic Activated Sludge

Two types of anaerobic activated sludge were used in the experiments described in this chapter as inocula for investigating the anaerobic degradation of the MWF. It is well known that there are only two types of anaerobic activated sludge employed in wastewater treatment: flocculent and granular sludge. Granular sludge is originally formed from flocculent sludge in reactors. It has a unique structure in that methanogens are protected inside the granular grains, and the microbial community in the sludge also changes during the granulation process (Lettinga et al., 1980; Kim et al., 1989; Lettinga, 1996; Hulshoff Pol et al., 2004). In this study, these two types of sludge were employed to represent the different microbe communities for investigating the anaerobic degradation of the MWF.

The flocculent anaerobic activated sludge was collected from a sludge digester at Thames Water Oxford Sewage Works (Thames Water Utilities Ltd.). The flocculent sludge sample was kept in a sealed bottle for about one month until no more biogas was generated from the sample. Then the Settled Sludge Volume (SSV) was adjusted to 50%, which means the volume of settled sludge was 50% of the total volume after 30 minutes of settling. Other major parameters of the sludge sample include: Mixed-Liquor Suspended Solid (MLSS) =28.61±0.35 g/L; and Volatile Suspended Solid (VSS) = 16.62±0.18 g/L. The granular sludge was collected from a full-scale Up-flow Anaerobic Sludge Blanket (UASB) reactor in a pharmaceutical wastewater treatment plant (Yufeng Pharmaceutical Ltd, Hebei, China). The range of the granular diameter was 1~2 mm, and its VSS/MLSS ratio was 0.95.

Figure 3.1 shows the visual differences between the flocculent sludge and the granular sludge samples.

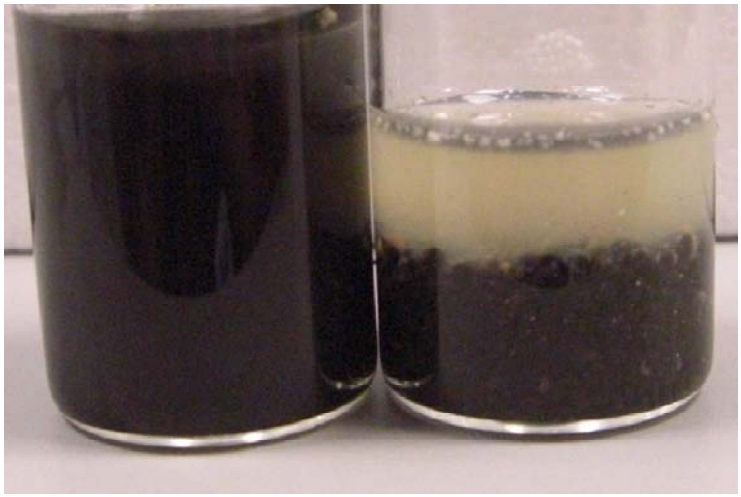

Figure 3.1: The flocculent sludge sample (left) and the granular sample (right) used in the study.

The experiments employing the sludge samples used 35℃ as the optimal experimental temperature, because the operating temperature which all the sludge samples were taken from was 35℃.

3.2.3 Co-Substrate

A nutrient broth solution (OXOID CM0001 Nutrient Broth, Oxoid, UK) was used in some of the experiments described in this chapter as a co-substrate. Its formula is given in Appendix 1.

3.2.4 Anaerobic Indicator

Resazurin (7-hydroxy-3Hphenoxazin-3-one-10-oxide, RNO; Acros Organics) was used as the chemical indicators of anaerobic condition in terms of reduction-oxidation potential (Redox Potential) in culture media and experimental systems. It is pH dependent and becomes colourless when it is kept in anaerobic conditions. In aerobic conditions, it is red (acidic condition), purple (neutral condition) or blue (alkaline condition). (Hungate, 1950; Balch, et al., 1976; Fukushima et al., 2002).

Resazurin was added in all the experimental systems described in this chapter at 1mg/L in order to indicate and control the anaerobic condition in the experiments. The COD value of the resazurin solution at 1mg/L was too low to affect the COD estimates, so it was ignored in all experiments. The photographs below show the change in the colour of resazurin in a bottle reactor freshly inoculated with activated sludge. The picture on the left in Figure 3.2 shows the colour of the bottle reactor system just after adding a fresh sample; the purple colour indicates the existence of oxygen in the reactor. The picture on

the right shows the colour of the same bottle reactor, which was maintained in a water bath for two hours after adding a fresh sample. It can be seen that the resazurin had changed from being purple to colourless, which indicates that the environment in the reactor had become strictly anaerobic.

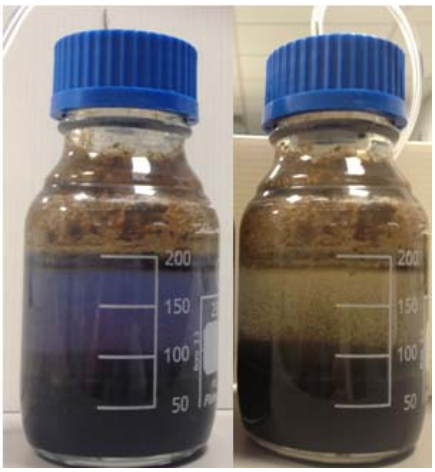

Figure 3.2: The colour of resazurin in a sludge reactor just after adding a fresh sample (left: pink) and that in the same reactor incubated in a water bath for 2 hours after adding a fresh sample (right: colourless).

3.2.5 Analytical Methods and Statistics

Determinations of COD, pH, MLSS, VSS, and TOC (see Abbreviations) in this study were made in accordance with the Standard Methods for the Examination of Water and Wastewater (APHA, 1992). The COD concentrations were measured using Hach Lange COD test cuvettes with a high temperature thermostat HT 200 S and a DR 2800 Spectrophotometer (Hach Lange, UK). The BOD concentration was measured with the test kit from Hach Lange which included a standard LCA555 BOD, LZC555 inoculation material and a LZC901 dilution water set. The pH values were measured using a JENWAY ion meter (3340/3345; JENWAY, UK). Biogas composition analysis was

carried out with a Shimadzu GC-TCD&FID (gas chromatograph with thermal conductivity detector and flame ionisation detector, GC 2010 series; Shimadzu, UK) fitted with an Agilent HP-PLOT/U column (0.53mm×30m; Agilent, UK).

Several micrographs of the flocculent activated sludge and the granular sludge were taken using a scanning electron microscope (SEM, JEOL 6500, JEOL, Germany) in the Department of Materials at the University of Oxford. The samples of the sludge were fixed with ethanol and coated with carbon particles (Jin, et al., 2007), please see Appendix 8 for details.

All the experiments described in this chapter were carried out in triplicate, including all control samples and control experiments. The results of the experiments are shown in figures with the mean values of the three replicates and error bars. All results of the calculations given in this chapter are rounded off to the first decimal. ANOVA test was used to determine the differences in the data to be contrasted.

3.2.6 pH Value of the MWF

As a typical water-soluble MWF, RELUBRO 800 BIO emulsion contains alkaline reserve agents to ensure that the pH of the MWF is restricted to a pH range of 8 to 9. Because of the high alkalinity of the MWF, its pH value may change significantly according to the dilution rate. Researchers have reported that anaerobic bacteria were sensitive to pH change, especially the methanogens (Sandin et al., 1990; Cheng et al., 2004; Perez et al., 2006). Thus, it was important to investigate the changes of the MWF's pH values at different dilution rates.

Two standard pH curves were produced in this experiment. One is the curve showing the changing pH value of the MWF sample diluted with de-ionised (DI) water. The other one is the curve showing the pH adjustment of a 100ml MWF sample using 0.2M/L HCl.

3.2.7 Factors Affecting the MWF's Anaerobic Degradation

1. Effect of Sample Concentration on Anaerobic Degradation of the MWF

It has been reported that the concentration of substrate(s) is one of the key environmental factors that can have significant effects on anaerobic degradation processes (Kim et al., 1992a; Perez et al., 2006). This experiment was set up to investigate the effect of the MWF's concentration on anaerobic degradation and to determine the optimal concentrations of the MWF for subsequent experiments.

The MWF samples were diluted to five concentrations: 10%, 30%, 50%, 70% and 100% v/v, and the pH adjusted to 7 in all cases. Fifteen millilitres of each MWF sample were kept in a sealed culture tube (25 ml) with 0.5 g dry anaerobic flocculent sludge with 1 mg/L resazurin. The culture tubes were flushed with N_2 gas for 10 minutes before sealing until the resazurin became colourless, and maintained in a shaking incubator at 35℃ for seven days. The COD reduction of the MWF samples and methane concentration in the tubes at the start and end points were determined. Two groups of control samples at each concentration were prepared, including the samples containing autoclaved dry sludge (autoclaved inoculum controls) and dry sludge samples with PBS buffer as a substrate (PBS controls).

2. Effect of Initial pH Value on Anaerobic Degradation of the MWF

It has been reported that the pH value is one of the key factors that determines the rate of anaerobic degradation (Anderson & Yang, 1992; van Haandel & Lettinga, 1994; Noike et al., 2000; Dhaked et al., 2010). Moreover, van der Gast et al. (2004) carried out an investigation into the effect of pH value on the aerobic degradation of spent MWFs inoculated with specific aerobic bacterial consortia, wherein it was concluded that the optimal pH range for biological treatment was between 6 and 7. However, whether the optimal pH range is the same with an anaerobic inoculum needs to be considered. The aims of this experiment were to investigate the effect of the initial pH of MWF samples on the anaerobic degradation of the MWF and the optimal pH range for subsequent studies.

The pH values of the MWF samples used in the experiment were adjusted to seven values at 5,000mgCOD/L using 0.2 M HCl and DI water. The final pH values of the samples were 4.96, 5.91, 6.59, 7.10, 7.35, 7.89, and 9.04. The sample set-up was the same as that of the experiment described in section 3.2.7.1. The blank samples at each pH value with added autoclaved sludge were prepared. The COD reduction of the MWF samples and methane concentration in the tubes at the start and end points were determined.

3.2.8 The Flask-Scale Reactor System Experiments

The experimental method described in section 3.2.7 aimed to determine the anaerobic degradation of the MWF samples in order to deduce their anaerobic degradability. However, there were three weaknesses in the experimental method, which may have caused some problems in regard to the accuracy and conclusions. Firstly, the experimental set-up did not allow an acclimatisation period for microbial communities in

activated sludge to adapt to the environment in the MWF samples, such as the degree of toxicity and pH. This may have caused a shock effect on the activity of the microorganisms, especially the methanogens. Secondly, according to Kim et al.'s studies (1989; 1990), some of the organic pollutants may be absorbed by the sludge, which would cause a significant error in relation to the evaluation of biodegradability, and this method had no way to avoid the error or to reduce its effect. Thirdly, degradation of the MWF sample changes the conditions in the tubes, such as the pH value and pressures in the tubes. The effects of the changes are not easily accounted for with this experimental set-up as detailed. Thus, a flask-scale reactor system was designed and a batch operation strategy employed for investigating the anaerobic biodegradability of the MWF.

1. Flask-Scale Reactor System and Batch Operation Strategy

The aims of employing the experimental design shown in Figure 3.3 with a batch operation strategy were to avoid the weaknesses of the method described in section 3.2.7 and to investigate the anaerobic degradation of the MWF by systematically using a standardised system that enabled comparison between experimental conditions.

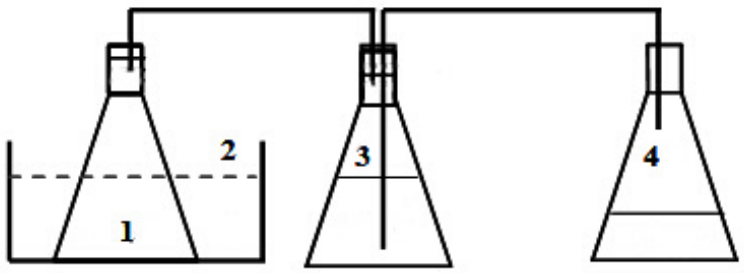

Figure 3.3: Design of the flask-scale anaerobic reactor system used in this study (1: Anaerobic Reactor. 2: Water Bath. 3: Biogas Collecting Bottle. 4: Water Collecting Bottle).

The experimental system had three parts: the reactor bottle, the biogas collection bottle and the biogas measurement bottle. The reactor bottle and biogas collection bottle were completely closed and airproof. The biogas collection bottle was filled with deionised water, which was gradually replaced and pushed into the water collecting bottle by the biogas produced in the reactor bottle. The volume of biogas production in the reactor was determined by measuring the volume of water in the water collecting bottle. The experimental system was set in triplicate in experiments.

The reactors were the same standard glass bottles with a total volume of 250ml and a working volume of 200ml. The reactors were seeded with 200ml flocculent anaerobic activated sludge or 100ml granular sludge (described in section 3.2.2) with a 100ml PBS solution. A semi-continuous operation strategy was employed with the reactor experiments, which entailed that the concentration of the MWF in the reactors increased gradually. The details of the operation strategy are given in the following sections regarding to the method of each reactor experiment.

The experimental method employed in section 3.2.7 was designed to investigate the substrate's anaerobic degradation by analysing the differences in terms of COD concentration and methane production between the start point and end point of the experiments. Its major weakness was that the experimental conditions were changed by the addition of the MWF and by the anaerobic degradation process itself. Thus, the results obtained from the experiments described in section 3.2.7 were only suitable for an initial investigation regarding the experimental factors for subsequent studies, but did not reach the accuracy level needed for a systematic investigation of the MWF's anaerobic

degradation. The research method employing the flask-scale reactor system and a semi-continuous operation strategy aimed to avoid or minimise the effects of the addition of a fresh MWF sample to the experimental system and the degradation of the MWF, and to maintain steady experimental conditions for investigating the anaerobic degradation of the MWF. The data was collected when the experimental system reached a "steady status".

According to the final results from the experiments employing the flask-scale reactor systems (see section 3.3.3 for details), this experimental method had three advantages, compared to the method described in section 3.2.7.

Firstly, a semi-continuous batch operation strategy was employed, which entailed that the concentration of the MWF sample in the reactors increased gradually. This provided time to allow the microorganisms to adapt to the environmental conditions in the MWF and to avoid the toxic and chemical shock effects caused by the constituent parts of the MWF. Secondly, according to the results of the experiments, the experimental method was able to maintain a steady pH value in the reactor systems during the operation period. This could avoid or minimise the errors caused by a fluctuating pH, because the high pH of the MWF sample was neutralised by the treated effluent in the reactor system. Thirdly, the experimental method was able to avoid the adsorption effect of the sludge on the COD reduction efficiency by analysing the data collected when the experimental systems reached a balanced/steady status. Any significant changes caused by the adsorption effect of the sludge on the organic components of the MWF, such as reaching the full

adsorption capability, could be reflected in the daily operation data and would alter the balanced/steady status of the experimental systems.

2. Key Definitions and Calculations of Operation Strategy

The semi-continuous operation strategy employed with the flask-scale reactor system is described below:

On each sampling occasion, 25ml of effluent water was sampled from the top of each reactor for analysis after allowing the sludge to settle down for 1 hour, and a 25ml fresh sample was added to replace the effluent sample. The final results were obtained through analysing the operation data in the period when the reactors reached "steady status". In this study, "steady status" was defined as the operational condition attained by a reactor when the daily effluent COD and the daily biogas production were relatively consistent, i.e. within 5% for more than three consecutive days. This concept was used to describe how the reactors recover their balance after the turbulences caused by adding the MWF samples.

The calculations of two key operation factors – hydraulic retention time (HRT) and daily COD removal efficiency – are highlighted in this section.

The HRT of the experiments was calculated according to the equation below (Eq. 3.1). When the magnitude of Vr is less than 1%, n is the HRT of the experiment.

$$\text{HRT} = n \quad (Vr \leq 0.01) \qquad \text{(Eq. 3.1)}$$

$$Vr = \left(\frac{Total\ Volume\ of\ Raw\ Liquid\ in\ reactor - Volume\ of\ Sampling}{Total\ Volume\ of\ Raw\ Liquid\ in\ reactor}\right)^{n}$$

Vr: volume of raw water remaining in a reactor
n: the operation time (day)

According to the operation strategy described above, the HRT of the experiments described in this section was around 16 days.

According to the reactor operation strategy, the daily COD removal efficiency of a reactor system was calculated with the following equation (Eq. 3.2):

$$E_R = \frac{COD\ Removed}{Total\ Raw\ COD} \times 100\% = \frac{Total\ Raw\ COD - Remaining\ COD}{Total\ \ COD\ of\ MWF\ sample\ added} \times 100\%$$

$$= \frac{C_{RB} \times V_R + C_S \times V_S - C_{RA} \times V_T}{C_S \times V_S} \times 100\% \qquad \text{(Eq. 3.2)}$$

E_R: Daily COD removal efficiency of the sludge reactor system
C_{RA}: COD concentration of the sample taken from the reactor system
C_{RB}: COD concentration of the sample taken from the reactor system on last sampling day
C_S: COD concentration of the MWF sample added into the reactor system
V_T: Total volume of the liquid in the reactor system before sampling
V_R: Volume of the liquid remaining in the reactor system after taking a sample
V_S: Volume of the MWF sample added to the reactor system
Note: C_{RA}=C_{RB} in a steady operation state

The final methane conversion rate of the biodegraded COD of a reactor system in the steady state was calculated with the equation below (Eq. 3.3):

$$E_M\ (L/g) = \frac{Volume\ of\ Methane\ Production}{COD\ Removed} = \frac{V_M \times C_M}{V_S \times (C_S - C_R)}$$

(Eq. 3.3)

C_S: COD concentration of the MWF sample added into the reactor system
C_R: COD concentration of the treated sample taken from the reactor system
V_S: Volume of the MWF sample added into the reactor system
V_M: Volume of the methane production
C_M: Concentration of the methane produced

3. Anaerobic Degradation of the MWF in the Flocculent Sludge Reactor System

The aim of the study was to investigate the anaerobic biodegradability of the MWF using the flask-scale reactor system inoculated with flocculent sludge.

Each reactor system was inoculated with 200 ml flocculent activated sludge (See section 3.2.2 for details). On each sampling occasion, 25ml of effluent water was sampled from the top of each reactor for analysis after allowing the sludge to settle down for 1 hour, and a 25ml fresh sample was added to replace the effluent sample. The daily operation data of effluent COD, pH and volume of biogas production were measured and recorded. Resazurin (1mg/L) was added to the bottle reactors as an indicator of the prevailing anaerobic conditions.

Three replicate reactor systems were prepared, and a group of control reactors with three replicates was set and operated with co-substrate only (5,000 mg/L, pH 7.2).

4. Anaerobic Degradation of the MWF in the Granular Sludge Reactor System

The aim of the experiment was to investigate the anaerobic biodegradation of the MWF sample using the flask-scale reactor inoculated with granular sludge. This experiment was carried out with the sample flask-scale bottle reactors, the MWF sample, and the operation and sampling strategies described in section 3.2.8.3. Three replicate flask-scale bottle reactors were inoculated with 100ml granular sludge and a 100ml PBS buffer. A group of control reactors with three replicates was set and operated with co-substrate only (5,000 mg/L, pH 7.2).

The results of this study were compared with the results of the flocculent sludge reactor systems described in section 3.2.8.3. After the experiment, the sludge samples were taken from the two types of reactors for SEM observation and pictures were taken for comparison. The two types of sludge samples were taken from the experiment system for

methanogen community analysis using the FISH probe method (See Chapter 4 for details).

3.3 Results and Discussion

3.3.1 pH Value of the MWF and Sample Dilution

The pH value of the substrate is a significant factor that needs to be considered in the biological degradation process of the substrate, especially in relation to the anaerobic degradation process (Bhattacharya & Parkin, 1989; Borja et al., 1996b; van der Gast et al., 2004). On the other hand, the alkaline reserve compounds in MWFs cause high pH, ranging from 8 to 11, with high alkalinity (Rossmoore, 1981; Sandin et al., 1990). Thus, it is important to investigate the pH change of the MWF sample used in this study when it is diluted and the pH adjusted.

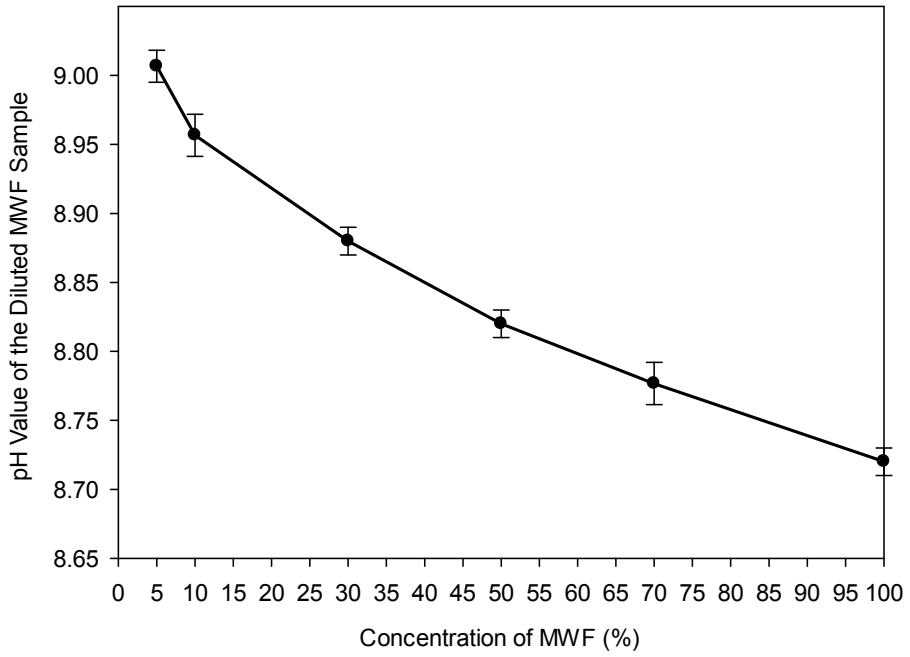

Figure 3.4: pH changes of the MWF sample diluted to decreasing concentrations (mean±SD of triplicates shown).

Figure 3.4 shows the pH values of the RELUBRO MWF samples diluted in six concentrations (100%, 70%, 50%, 30%, 10% and 5%) with DI water. It can be seen that

the pH value of the MWF sample increased from 8.7 to 9, roughly, with decreasing concentration or an increasing dilution rate. Figure 3.5 below shows the standard curve of the adjusted pH value of the MWF using a 0.2M HCl solution. It can be seen that the pH changes of the MWF sample and the usage of acid were intertwined, although without a linear progression. The results shown in Figure 3.4 and Figure 3.5 indicate the effect of the alkaline reserve in the sample pH changes. However, these results need to be interpreted with caution to avoid any error regarding the pH change of the MWF sample in this study. Furthermore, the results indicate that it is important to carry out the subsequent experiments at a stable pH. Dilution of the MWF sample may reduce the toxicity of the MWF, but the high pH caused by dilution could also have an inhibitory effect on anaerobic microorganisms in experimental systems. These two effects should be investigated separately.

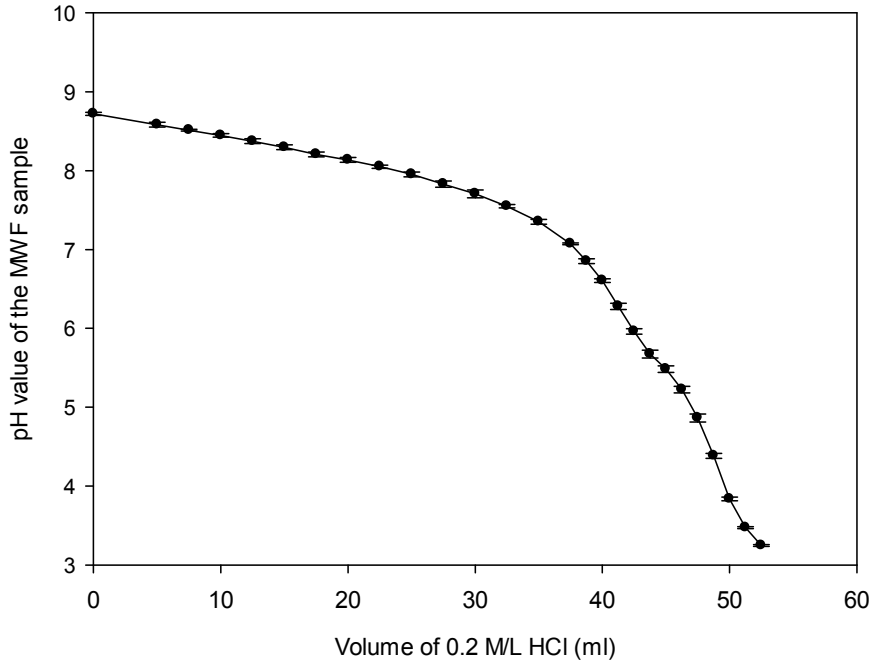

Figure 3.5: Standard curve of the adjusted pH value of the MWF sample using a 0.2M HCl solution (100ml MWF sample, mean±SD of triplicates shown).

3.3.2 Factors Affecting the MWF's Anaerobic Degradation

1. Effect of Sample Concentration on Anaerobic Degradation of the MWF

The literature review revealed that the concentration of a MWF is the most important factors affecting the anaerobic degradation process; it was reported in previous studies that high COD concentration was normally accompanied by high toxicity in MWFs (Kim et al., 1992a; Cheng, et al., 2005). Thus, it was important to determine the effect of sample concentration on the anaerobic degradation of the MWF. This experiment aimed to determine if the MWF sample can be decomposed by anaerobic microorganisms with methane production, and to investigate the effect of the sample concentration on the anaerobic biodegradability of the MWF.

The MWF sample was diluted to five different concentrations with adjustment of pH to 7, and inoculated with flocculent sludge. The concentrations of methane produced in the test tubes were determined and analysed using the GC system with a FID detector (GC 2010 series; Shimadzu, UK). The results of the COD reduction of the MWF samples in seven days are shown in Figure 3.6 below.

Figure 3.6 shows that the COD reduction rates of the MWF samples treated with flocculent activated sludge increased with the decreasing concentration of the MWF sample. This finding confirms that the toxicity of the MWF sample at a high concentration inhibits the anaerobic degradation of the organic compounds in the MWF (Perez et al., 2006).

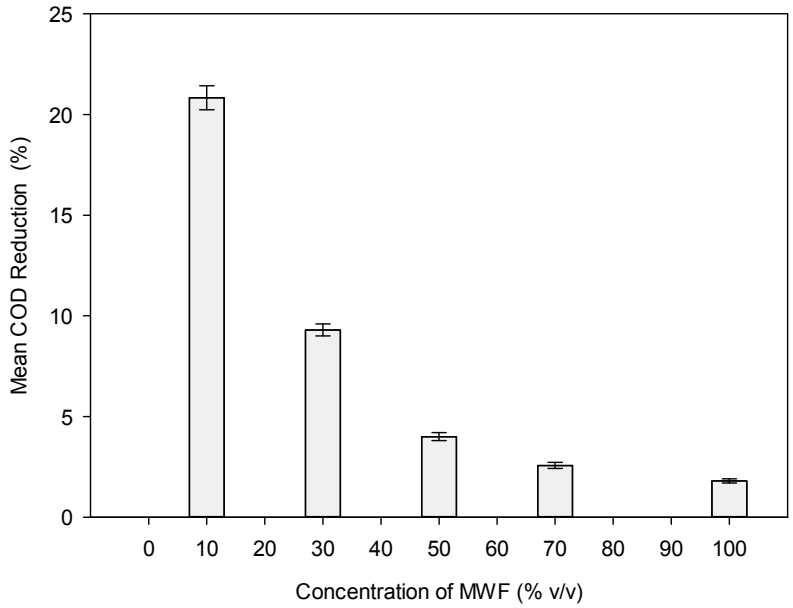

Figure 3.6: The mean COD reduction of the MWF samples in different concentrations treated with anaerobic sludge (7 days at 37 ℃, mean±SD of triplicates shown).

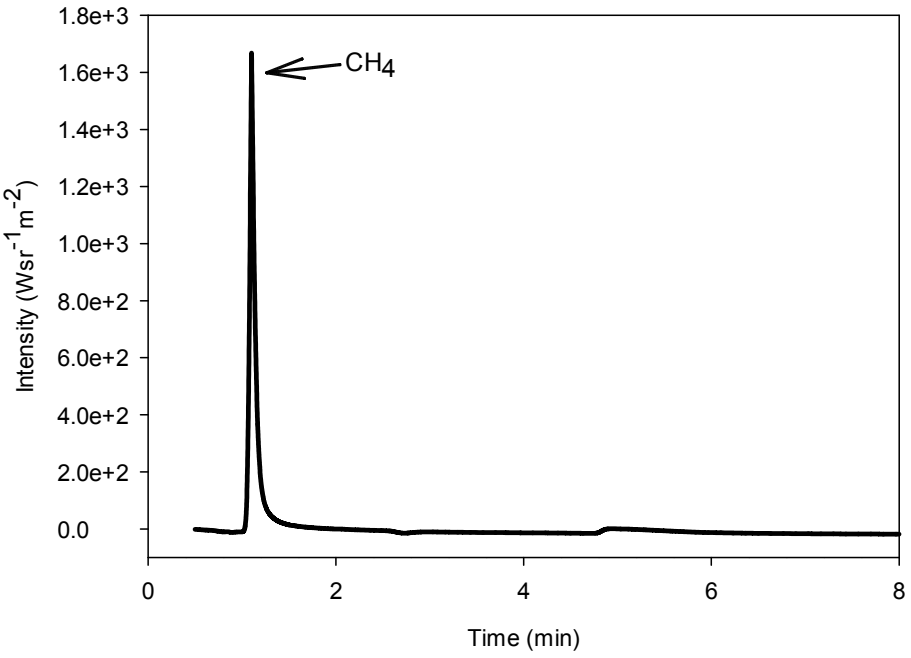

Figure 3.7: Methane detection of the top space in one of three replicate sealed culture tubes containing a 10% diluted MWF sample (10,000mgCOD/L, pH 7) and 0.5 g dry sludge inoculum after 7 days' incubation at 37℃ (GC-FID system).

There was no measurable methane production detected using the GC system from the MWF samples diluted to 50%, 70% and 100% of raw concentration (100,000mgCOD/L), while only a trace of methane was detected in the tubes containing the MWF samples diluted to 10% and 30% of raw concentration. The result of methane detection of a test tube is given in Figure 3.7 above, containing a 10%v/v diluted MWF sample (10,000mgCOD/L, pH 7) and 0.5 g dry sludge inoculum after seven days' incubation at 37℃; its methane concentration was roughly 11%. This confirmed that the semi-synthetic MWF used in this study was anaerobically biodegradable, and the toxicity of the MWF sample at high concentration inhibits the activity of methanogens significantly.

There was no detectable COD reduction and methane production from the autoclaved inoculum control samples which were inoculated with autoclaved sludge. The concentrations of methane produced from the PBS control samples containing activated sludge and the PBS buffer were too low to be measureable. Furthermore, there was no obvious COD reduction and methane production in any control sample, which indicates that all the COD reduction and methane produced from the MWF samples were due to the activity of the anaerobic microorganisms within the activated sludge, rather than the bacteria colonising in the MWF samples.

In summary, the results suggest that the RELUBRO 800 sample was anaerobically biodegradable, and the methane was produced in the degradation process of the MWF. In addition, considering the toxicity of the MWF, it was established that the concentration of the MWF sample selected for subsequent experiments should be at a relatively low: 5,000 mgCOD/L (5% of raw concentration).

2. Effect of Initial pH Value on Anaerobic Degradation of the MWF

The pH of the substrate is one of the most important factors to be considered when carrying out studies of anaerobic degradation, because a high pH may cause inhibitory effects on anaerobic microorganisms, especially methanogens. Selecting the optimal pH range for carrying out the experiments is also important when investigating methane production from wastewater (Hansen et al., 1999; Noike et al., 2000; Cheng et al., 2005).

Figure 3.8 shows the differences in mean COD reduction rates of the MWF samples treated with activated sludge at the same concentration but at different initial pH values. Figure 3.9 below shows the volume of methane production from the MWF samples at different initial pH values.

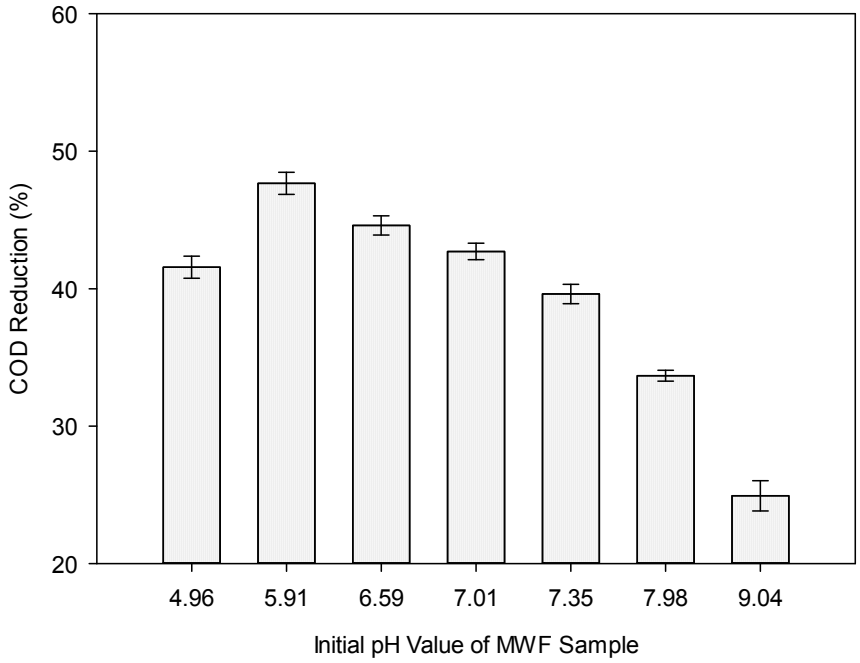

Figure 3.8: Mean COD reduction of the MWF sample (5,000mg/L) at different initial pH values treated with activated sludge (7 days, n=3, error bar: SD).

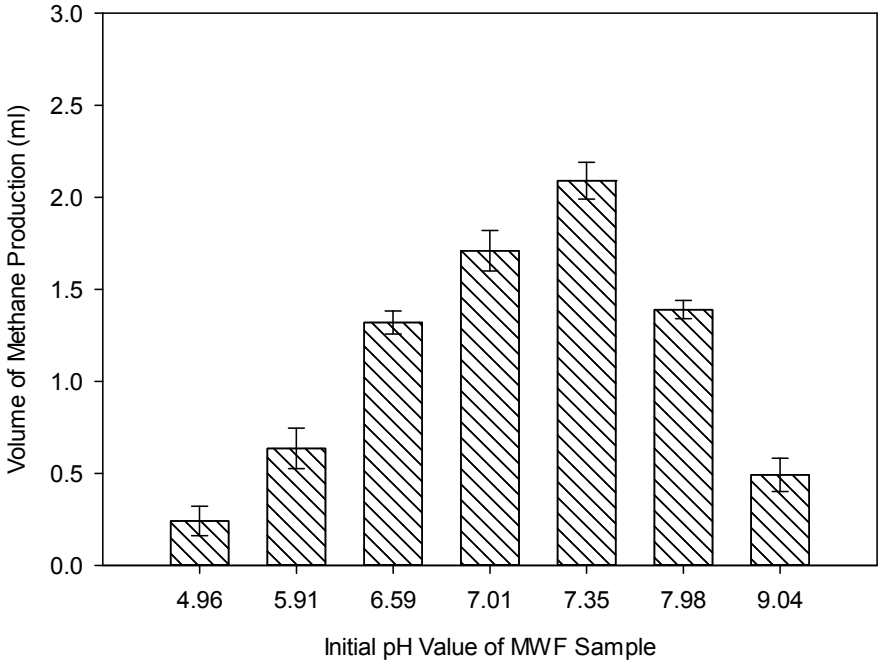

Figure 3.9: Mean methane production from the MWF (15ml, 5,000mgCOD/L) at different initial pH values treated with activated sludge for 7 days (n=3, error bar: SD).

Table 3. 4: Results of ANOVA for the data shown in Figure 3.8 and Figure 3.9

Source of Variation	DF	SS	MS	F	P
Data in Figure 3.8					
Between Groups	6	1060.04	176.67	309.95	<0.001
Residual	14	7.98	0.57		
Total	20	1068.02			
Data in Figure 3.9					
Between Groups	6	8.40	1.40	177.77	<0.001
Residual	14	0.11	0.01		
Total	20	8.52			

The results of ANOVA test for the data given in these two figures are given in Table 3.3 below. The differences in the mean values among the groups are greater than would be expected by chance; there are statistically significant differences (P = <0.001) in both groups of data.

Figure 3.8 and Figure 3.9 show that the samples with initial pH values between 5 and 7 had a higher COD reduction rate, but the initial pH values of the samples which had a greater level of methane production were roughly in the range of 7 to 7.3. These results are consistent with those of other studies and suggest that the optimal pH for investigating the biodegradation of MWFs is around 7 (Cheng et al., 2005; der Gast et al., 2004). Subsequent studies on the anaerobic degradation of the MWF in a neutral pH range (pH 7 to 7.3) are therefore recommended.

3.3.3 The Flask-Scale Reactor System Experiments

1. Anaerobic Degradation of the MWF in the Flocculent Sludge System

Figure 3.10, Figure 3.11, Figure 3.12 and Figure 3.13 present the experimental data of the flask-scale bottle reactor systems inoculated with flocculent activated sludge.

Figure 3.10 shows the COD concentration of the influent samples and the changes in the mean effluent COD concentrations in the flocculent sludge reactors fed with the MWF sample and the control reactor systems over the 20-day operation.

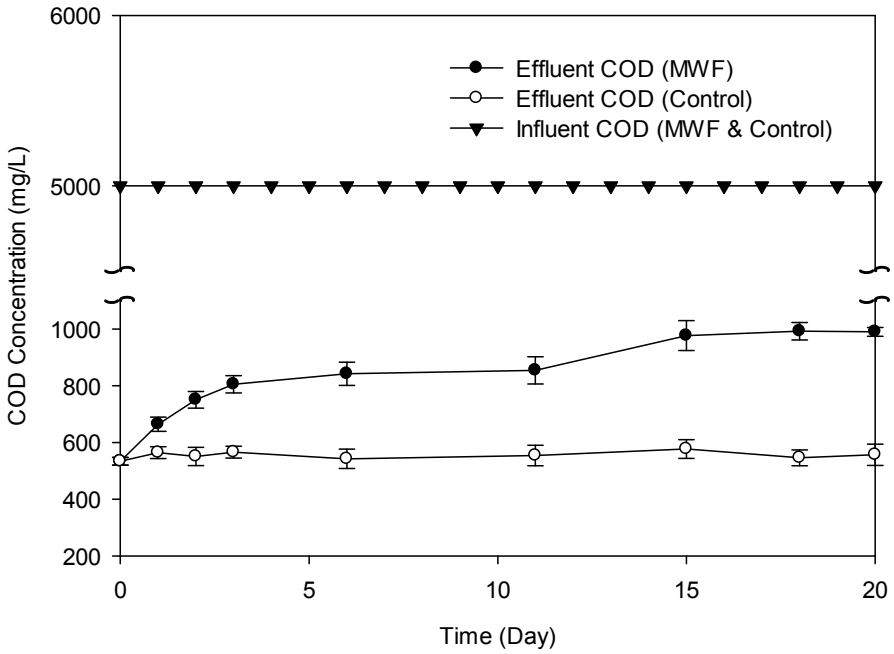

Figure 3.10: Changes in the mean effluent COD concentration of the flocculent sludge reactors fed with MWF sample (5,000mgCOD/L, pH 9.6) and control reactors fed with co-substrate (nutrient broth, 5,000mgCOD/L, pH 7.2) over the 20-day operation (n=3, error bar: SD).

As can be seen from Figure 3.10, the COD concentration in the reactors fed with the MWF sample increased gradually, while the co-substrate solution in the reactors was replaced by the MWF sample. The mean effluent COD concentration reached around

1,000mg/L from around 500mg/L. The differences shown with the error bars in the figure were caused by the differences in the system set-up. According to Equation 3.2, the final COD removal efficiency of the experiment was 80.1±0.8 %, and the COD removal efficiency of the reactors in the last four days of the operation was within 5%, which indicates the results were consistent. In contrast to the changes in the effluent COD concentration in the reactors fed with the MWF, there was no significant change in the mean effluent COD concentration of the control reactors during the 20-day operation with the COD removal efficiency of the control reactors being 90.4±1.5%.

Figure 3.11 below shows the changes in the effluent pH of the reactors fed with the MWF sample and control group over the 20-day operation.

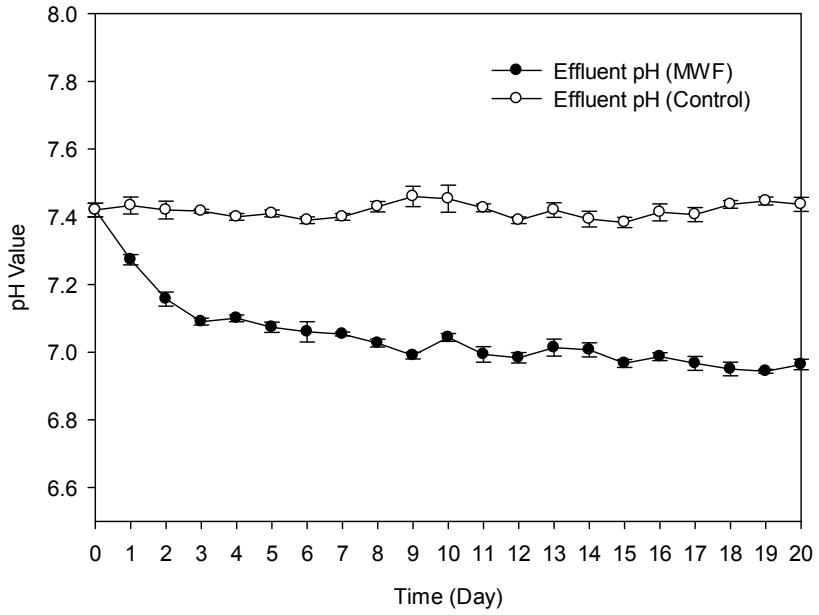

Figure 3.11: Changes in the mean effluent pH value in the flocculent sludge reactors fed with the MWF sample (5,000mgCOD/L, pH 9.6) and the control reactors fed with co-substrate (nutrient broth, 5,000mgCOD/L, pH 7.2) over the 20-day operation (n=3, error bar: SD).

Figure 3.11 illustrates that, as a consequence of feeding with the MWF sample, the

system pH value in the reactors fell dramatically from 7.4 to 7.1 in the first three days, then slowly reduced to around 7 in another 12 days. However, no significant pH change appeared in the control reactors fed with the co-substrate solution. Considering that the pH value of the influent MWF sample was 9.2, the results suggest that this experiment design and operation strategy maintained the system pH of the reactors and successfully reduced the effect of the changing pH on the investigation of the anaerobic biodegradability of the MWF. The pH drop suggests a significant production of acid breakdown products in the anaerobic degradation process of the MWF, which neutralised the high pH of the fresh MWF sample and also reduced the pH.

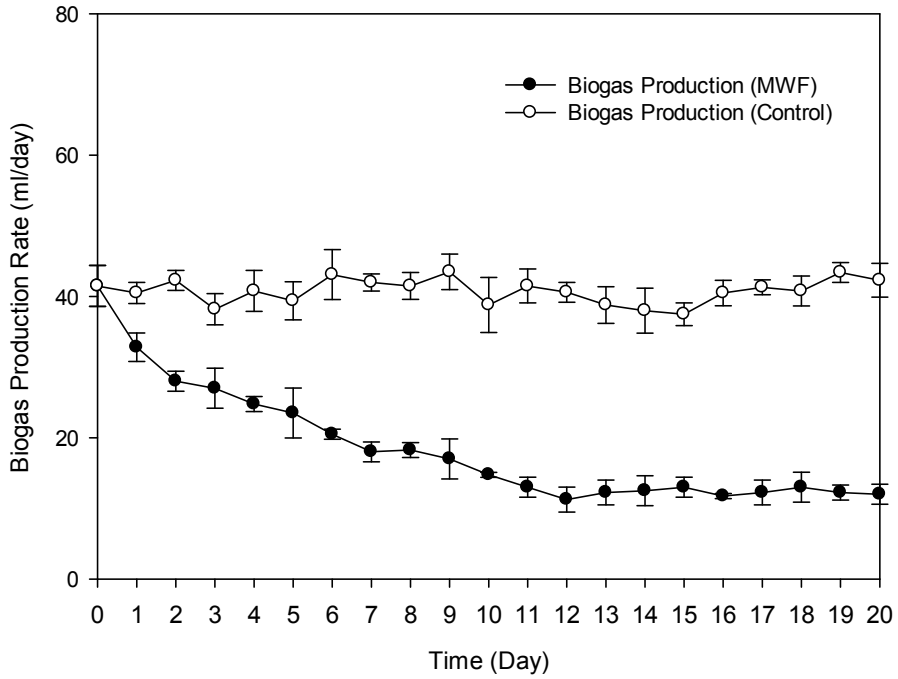

Figure 3.12: Changes in the mean volume of daily biogas production in the flocculent sludge reactors fed with the MWF sample (5,000mgCOD/L, pH 9.6) and the control reactors fed with co-substrate (nutrient broth, 5,000mgCOD/L, pH 7.2) over the 20-day operation (n=3, error bar: SD).

Figure 3.12 presents the changes in the mean volume of daily biogas produced from the

flocculent sludge reactors. It can be seen from Figure 3.12 that, the mean volume of daily biogas production reduced substantially from 41.5ml to 12.8ml and remained stable. The differences shown with the error bars in the figure were caused by the differences in the sett-up of the systems.

Figure 3.13 shows the result of the biogas composition analysis (GC-TCD) of a biogas sample taken from one of the flocculent sludge reactors fed with the MWF sample on the last day of the 20-day operation.

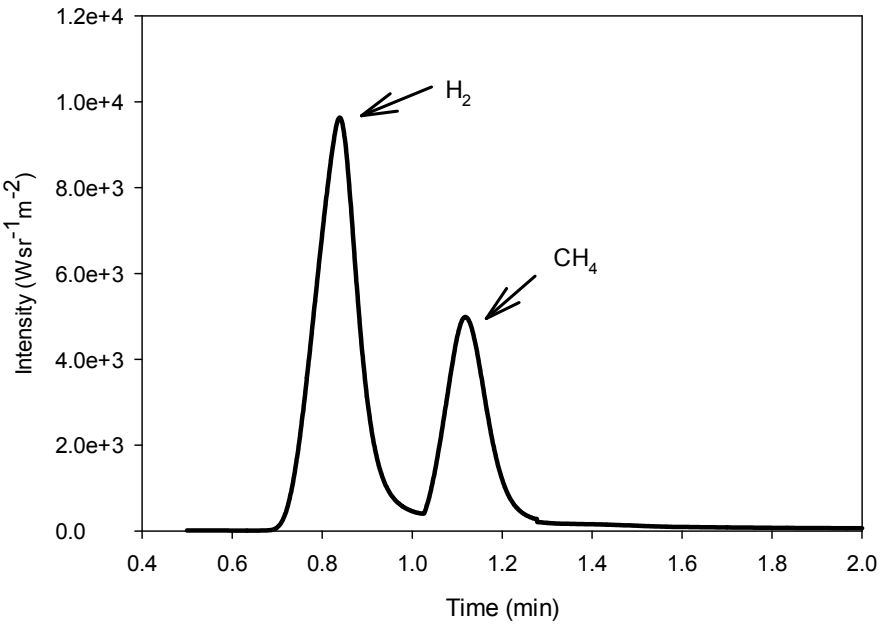

Figure 3.13: Biogas composition analysis of the biogas generated from a flocculent sludge reactor system operated with MWF sample (5,000mgCOD/L, pH 9.6) on the 20th day of the 20-day operation using the GC-TCD system.

The mean concentration of methane in the biogas generated from the flocculent sludge reactors on the last day (day 20) of the stable operation period was 45.6±1.2% whereas the concentration of the biogas generated from the control reactors was 61.2±2.1%. In total, the volume of daily methane production from the flocculent sludge reactors reduced

by 77%, more or less, after 20 days' operation with the MWF sample. The theoretical methane yield is 0.35L/g COD removed (Kim et al. 1992). According to Equation 3.3, the methane yield of the flocculent sludge reactor systems was around 0.058L/g, which is just around 17% of the theoretical value.

To sum up, as shown in the four figures (Figures 3.10−3.13), the results indicate that around 80% of the MWF was anaerobically biodegradable at 5,000mgCOD/L (pH 9.6) in the reactor system inoculated with flocculent sludge, while methane was detected in the biogas generated from the reactors. However, the results also suggest the anaerobic biodegradability of the MWF was lower than that of the co-substrate, and the decrease in the system pH value suggests that low-biodegradable or non-biodegradable components and acid breakdown products accumulated in the reactors during the 20-day operation with the MWF sample. The change in the COD reduction rates and the significant reduction in the volume of daily methane production from the reactors fed with the MWF, compared with the control systems, demonstrate that the MWF sample caused a significant inhibitory effect on the microorganisms in the anaerobic degradation system, especially on the activity of methanogens. Thus, an investigation using the experimental system inoculated with granular sludge was carried out as described in the next section. A comparison of the two systems' performance would indicate the possibility of enhancing the anaerobic degradation of the MWF by overcoming the toxicity of the MWF, through employing specific anaerobic bacterial strains.

2. Anaerobic Degradation of the MWF in the Granular Sludge System

As introduced in section 3.3.2, there are only two types of anaerobic activated sludge utilised in anaerobic wastewater treatment processes. Granular sludge is normally formed from flocculent sludge and its anaerobic microbes are selected naturally by operating with a specific organic wastewater during the granulation process (Hulshoff Pol et al., 2004). In this study, the two types of anaerobic activated sludge were selected to determine how the two systems performance would compare for treating the MWF.

Figure 3.14, Figure 3.15, Figure 3.16 and Figure 3.17 present the experimental data for the flask-scale bottle reactor systems inoculated with the granular activated sludge in the 20-day operation with the MWF sample (5,000mgCOD/L, pH 9.6).

Figure 3.14 shows the changes in the mean effluent COD concentration of the granular sludge reactors fed with the MWF sample (5,000mgCOD/L, pH 9.6) and the control group fed with co-substrate solution (nutrient broth, 5,000mgCOD/L, pH 7.2) over the 20-day operation.

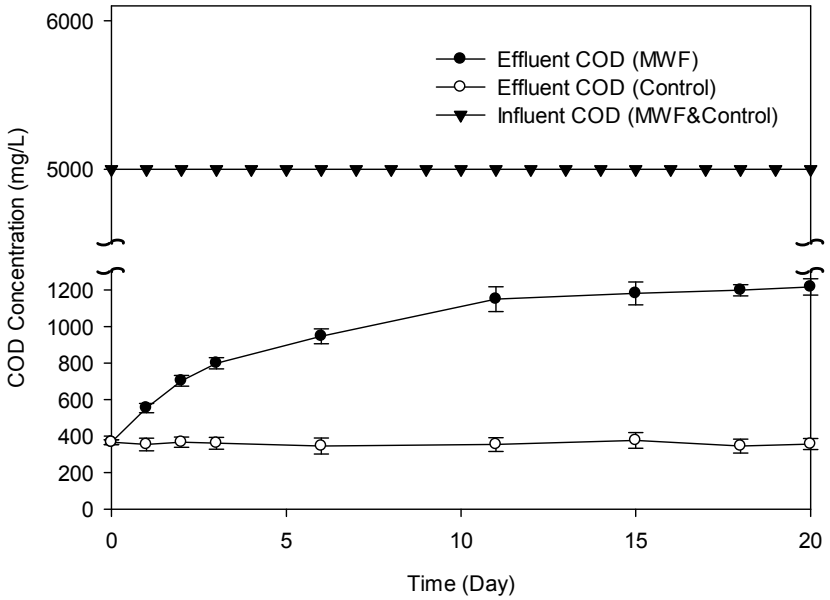

Figure 3.14: Changes in the mean effluent COD concentration of the granular sludge reactors fed with MWF sample (5,000mgCOD/L, pH 9.6) and control reactors fed with the co-substrate (nutrient broth, 5,000mgCOD/L, pH 7.2) over the 20-day operation (n=3, error bar: SD).

As can be seen from Figure 3.14, the COD concentration in the reactors fed with the MWF sample increased gradually while the co-substrate solution in the reactors was replaced by the MWF sample, as happened in the flocculent sludge reactors. The mean effluent COD concentration reached around 1,200mg/L from around 380mg/L. According to Equation 3.2, the final COD removal efficiency of the experiment was 76.3±1.4%, and the relative consistency of the COD removal efficiency of the reactors in the last four days of the 20-day operation was within 5%. In contrast to the changes in the effluent COD concentration in the reactors fed with the MWF, there was no significant change in the mean effluent COD concentration of the control reactors during the 20-day operation, with the COD removal efficiency of the control reactors being 92.4±0.3%.

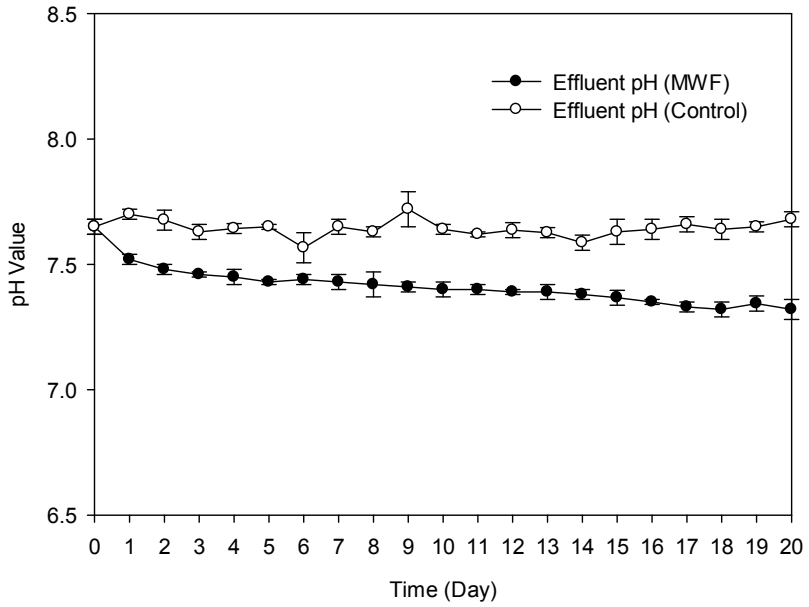

Figure 3.15: Changes in the mean effluent pH value of the granular sludge reactors fed with the MWF sample (5,000mgCOD/L, pH 9.6) and the control reactors fed with the co-substrate (nutrient broth, 5,000mg/L, pH 7.2) over the 20-day operation (n=3, error bar: SD).

Figure 3.15 shows the changes in the mean effluent pH value of the granular sludge reactors fed with the MWF sample and the control reactors over the 20-day operation. The pH values of the influent MWF sample and influent co-substrate sample were 9.6 and 7.2 respectively.

Figure 3.15 shows that the system pH value of the reactors fell gradually from 7.6 to 7.3 after the feeding of the MWF sample started, and then remained stable over the last days of the operation. On the other hand, no significant pH change appeared in the control reactors. The pH drop also suggests an accumulation of acid breakdown products in the anaerobic degradation process of the MWF, as was the case in the flocculent sludge reactors.

Figure 3.16 below presents the changes in the mean volume of daily biogas production of

the granular sludge reactors fed with the MWF and the control reactors over the 20-day operation. Figure 3.17 illustrates the result of the biogas composition analysis (GC-TCD) of a biogas sample taken from one of the granular sludge reactors fed with the MWF sample on the last day of the 20-day operation.

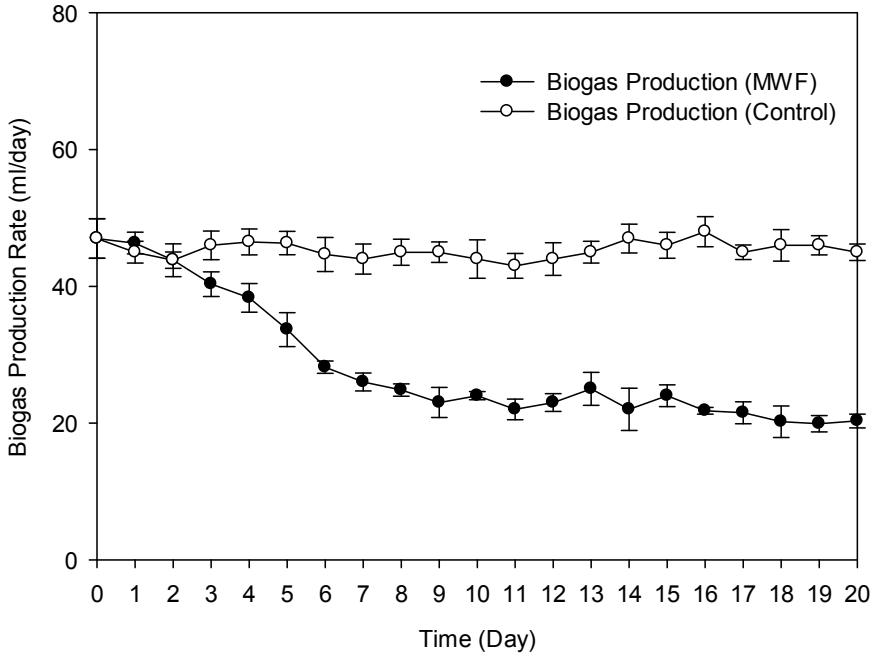

Figure 3.16: Changes in the mean volume of daily biogas production of the granular sludge reactors operated with the MWF sample (5,000mgCOD/L, pH 9.6) and the control reactors fed with co-substrate (nutrient broth, 5,000mgCOD/L, pH 7.2) over the 20-day operation (n=3, error bar: SD).

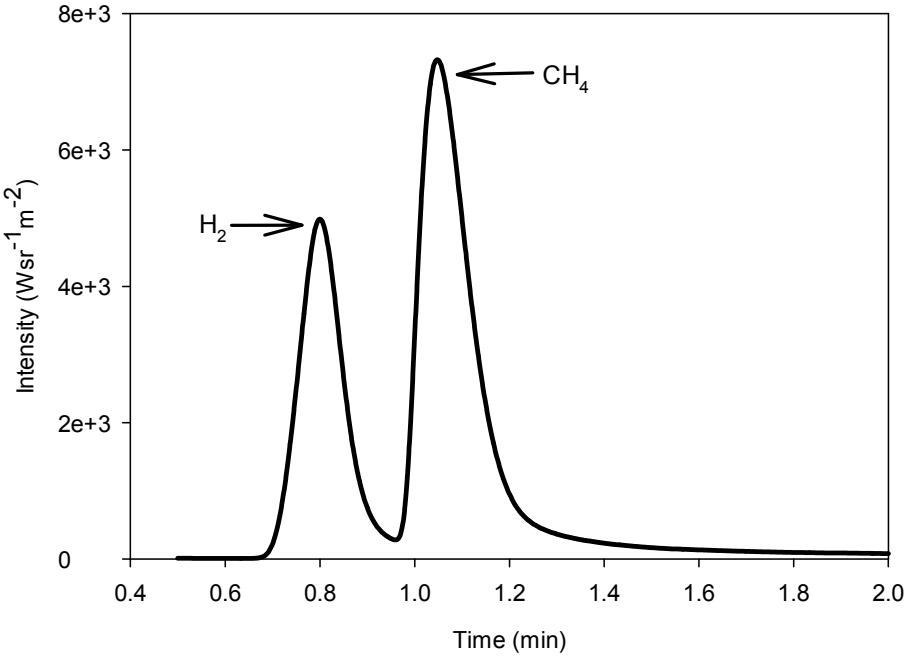

Figure 3.17: Biogas composition analysis of the biogas generated from a flocculent sludge reactor system operated with the MWF sample (5,000mgCOD/L, pH 9.6) on the 20th day of the 20-day operation using the GC-TCD system.

In Figure 3.16 and Figure 3.17, it can be seen that the mean volume of daily biogas production reduced substantially from about 46.1ml to around 21.3ml and remained stable. The differences shown with the error bars in the figure were caused by the differences in the system set-up. The methane concentration of the biogas from the reactors fed with the MWF was 56.2±2.3 % whereas the methane concentration of the biogas sample taken from the control reactors was 66.3±1.9%. In total, the volume of daily methane production from the granular sludge reactors reduced by 61%, more or less, after the 20-day operation with the MWF sample. The theoretical methane yield is 0.35L/g COD removed (Kim et al. 1992). According to Equation 3.3, the methane yield of the flocculent sludge reactor systems was around 0.124L/g, which is just around 35% of the theoretical value.

To sum up, the results confirmed that the MWF sample was anaerobically biodegradable and methane was produced in the granular sludge reactor system as well as the flocculent sludge system. Moreover, the accumulation of acid breakdown products and the strong inhibitory effect of the MWF on the anaerobic degradation also appeared in the granular sludge reactors. The results and data obtained from the experiments employing flocculent and granular sludge are compared in the next section for concluding on the toxic effect of the MWF on anaerobic microorganisms and estimating the possibility of employing specific anaerobic bacterial strains to treat the MWF in order to overcome the toxic effects.

3. Comparison of Flocculent and Granular Sludge Systems

The results of the flask-scale reactor systems inoculated with the two types of sludge demonstrate that the MWF, which in this case was a typical water-soluble MWF, was anaerobically biodegradable and the methane was generated in its degradation process. The results also demonstrate some similar significant phenomena in regard to the anaerobic degradation process of the MWF. Compared with the control group fed with nutrient broth solution, the experimental systems fed with the MWF sample had increasing COD concentration of the treated effluents, decreasing system pH and reducing biogas production in the early stage of the 20-day operation. Such common findings underline the reliability of the method and results. Moreover, the results of the two types of reactor systems also showed some noticeable differences, which may suggest some other significant inherent laws governing the anaerobic degradation process of the MWF.

A comparison of the results in this section reveals some significant differences in their performance regarding both degradation of the MWF sample and the generation of methane. These differences are probably due to the differences between the types of anaerobic activated sludge.

Figure 3.18 below illustrates the visual differences between the flocculent and granular sludge used in the flask-scale reactor experiments using SEM. The differences of the physical structures and microbial communities on the surface of the sludge samples were quite obvious due to the different mixtures of coccus and bacillus. This finding is in agreement with the findings of previous studies which showed a unique structure for granular sludge in that methanogens are protected inside of grains by hydrolytic and acid-forming bacteria (Lettinga et al., 1989; Anderson et al., 1991; Alphenaar et al., 1993, 1994; Abreu et al., 2007).

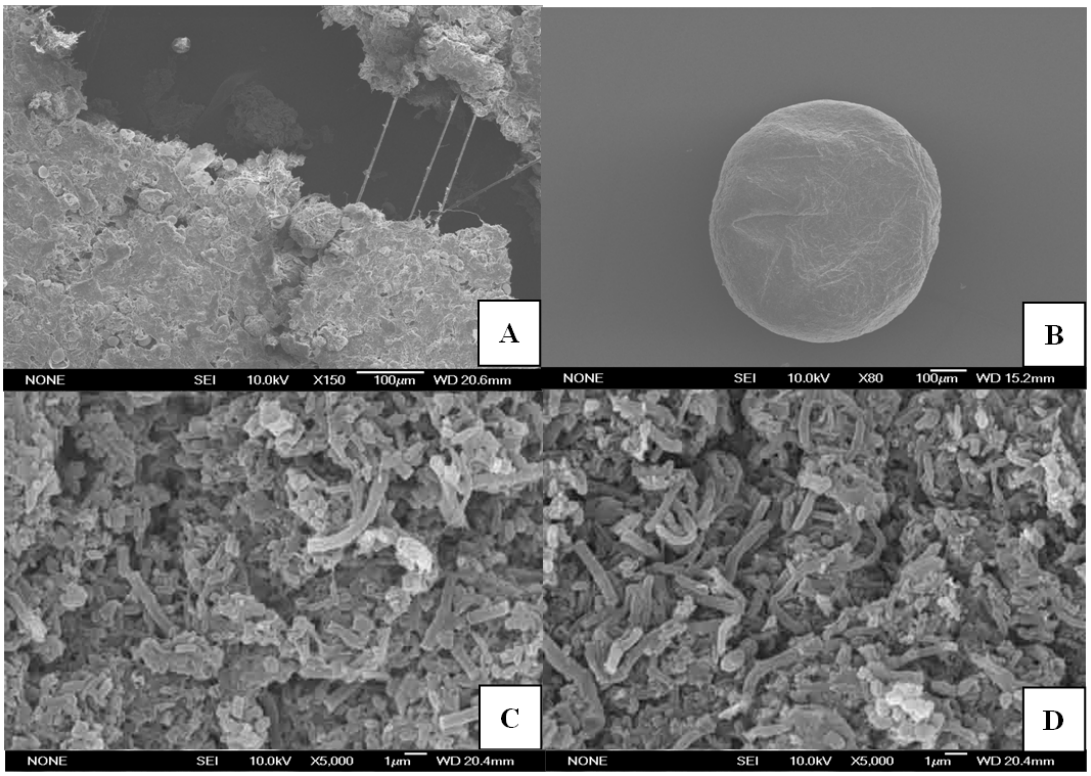

Figure 3.18: SEM pictures of flocculent sludge (A) and granular sludge (B) used in flask-scale reactor systems; the surface of flocculent sludge (C) and surface of granular sludge (D).

Figure 3.19 below shows a performance comparison of the two types of sludge reactors on the COD reduction rate and the daily methane production in the flask-scale reactor experiments. It can be seen that the mean COD reduction rate of the flocculent sludge reactors was about 5% greater than that of the granular sludge reactors, whereas the granular sludge reactors had a methane production rate two times higher than that of the flocculent sludge. Overall, approximately 80% of the MWF sample was found to be anaerobically biodegradable at 5,000mgCOD/L.

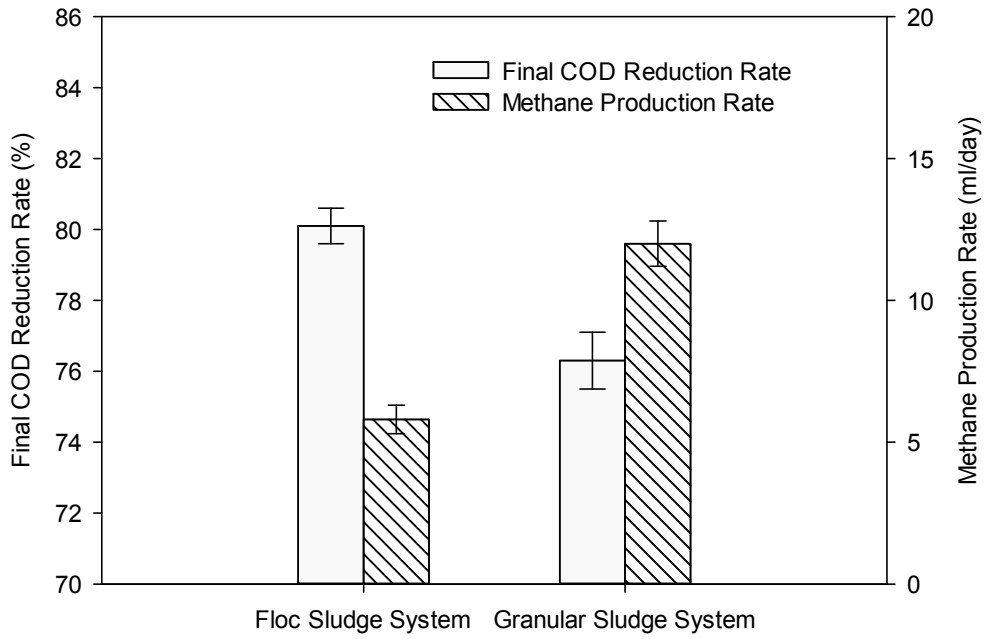

Figure 3.19: The mean COD reduction efficiency and volume of daily biogas production of the flask-scale reactor systems inoculated with flocculent and granular sludge in the final steady status of the 20-day operation (n=3, error bar: SD).

According to previous studies on the differences between flocculent and granular sludge, there are two possible explanations of these differences in the experimental results.

Firstly, even though the same sludge volume was set in each flask-scale reactor with the two types of anaerobic sludge, granular sludge normally has a much greater concentration of biomass than flocculent sludge, but less biodiversity because of the natural selection during the granulation process (Lettinga et al., 1989; Hulshoff Pol et al., 2004). It was likely that the larger methanogen population in the granular sludge enhanced the conversion from organic acid breakdown to methane, but the lower biodiversity of the granular sludge greatly limited the decomposition of the petroleum compounds in the MWF sample. Figure 3.20 shows the comparison of the pH changes in the two types of reactors during the 20-day operations. The higher overall pH value of the granular sludge

system suggests the greater methane production capability of the granular sludge using organic acid breakdowns.

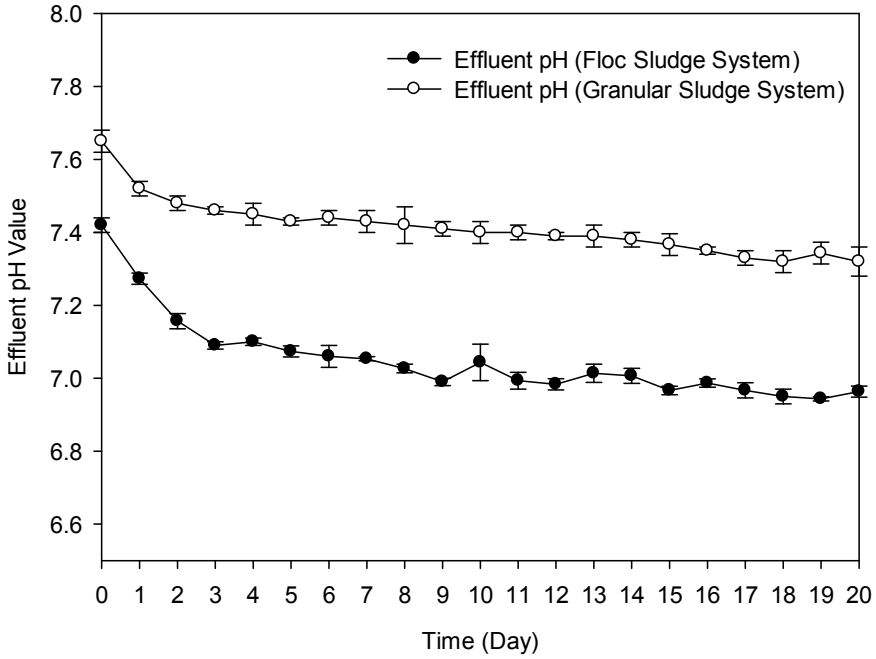

Figure 3.20: Comparison of the mean effluent pH of the flask-scale reactor system inoculated with flocculent and granular sludge during the 20-day operation with the MWF sample (n=3, error bar: SD).

Secondly, as introduced above, granular sludge has a unique structure in that most of the methanogens exist inside the grains and are protected by other anaerobic bacteria from toxic environments (Anderson et al., 1991; Alphenaar et al., 1993).

This study placed great emphasis on reducing the inhibitory effect of the MWF so as to enhance methane production in the anaerobic degradation process of the MWF. The significant differences in terms of methane production between the two types of anaerobic sludge were of particular interest in this study. The inhibitory effect of the MWF sample on the activity and community of methanogens was investigated in the studies described in Chapter 4. Most published studies took no note of the importance of

investigating the changes to the methanogen communities in the anaerobic treatment process of MWFs in terms of finding new methods to enhance the methane production process. Furthermore, the RELUBRO MWF, a typical water-soluble MWF, had relatively high biodegradability in the sludge systems, even though some significant inhibitory effects appeared. This also demonstrates a strong possibility that anaerobic bacteria can survive in MWFs. This suggests the high potential of employing isolated anaerobic bacteria in reducing the toxicity of MWFs, biologically, in terms of strengthening the anaerobic degradation process of MWFs and enhancing methane production in the degradation process.

3.4 Conclusions

The overall aim of the studies described in this chapter was to systematically develop an experimental method for the investigation of the anaerobic degradation of the MWF. Based on the previous studies reviewed and the outcomes of an initial investigation, a flask-scale reactor system was developed with a batch operation strategy. Two types of anaerobic activated sludge – flocculent and granular sludge – were employed in the experiments with the flask-scale reactors.

The studies presented in this chapter revealed the following major findings in regard to the removal of organics, expressed as COD reduction, and methane production in the anaerobic degradation process of the RELUBRO MWF:

I. Approximately 80% of the RELUBRO MWF sample was found to be anaerobically biodegradable in the treatment process employing anaerobic activated sludge at 5,000mgCOD/L (HRT 16 days).

   Methane gas was produced in the anaerobic degradation process of the MWF sample inoculated with anaerobic activated sludge; methane production can also be confirmed by the existence of methanogens in the sludge sample taken after the experimental operation (see Chapter 4 for details). Approximately 35% of the biodegraded COD could be converted to methane in the sludge reactor systems.

II. Influent COD concentration up to 5,000mg/L appeared to play an inhibitory role in regard to anaerobic microorganisms, especially methanogens.

III. To increase the removal efficiency of the waste organic matter and enhance methane production in the anaerobic treatment of MWFs, some methods should

be employed to reduce the inhibitory effect of MWFs or to improve the microbe communities. The findings of the studies in this chapter have important implications for developing an approach to reduce the inhibitory effect of MWFs on their anaerobic degradation process by employing the anaerobic bacteria isolated from spent MWFs.

In addition, two secondary findings can be concluded from the investigation of the MWF's anaerobic degradation and the comparison between the flocculent sludge system and the granular sludge system. These are as follows:

(1) The optimal pH value range for carrying out the anaerobic biodegradation of MWFs was between 7 and 7.35;

(2) The results obtained from the experimental systems inoculated with flocculent and granular sludge demonstrate some similar findings, including that the MWF was anaerobically biodegradable with methane production, and the MWF inhibited the anaerobic degradation process in terms of causing accumulation of acid breakdown products and reducing methane production at a fairly large scale. On the other hand, the comparison also shows some significant differences in the performance of the two types of experimental systems regarding both the COD reduction rate and methane production rate. It was concluded from the comparison that there was a great potential for enhancing the anaerobic degradation of the MWF by overcoming the toxicity effect of the MWF through improving the microbial communities.

A significant effect of the MWF on the anaerobic degradation process, especially on

methane production, was noticeable in the investigation of the MWF's anaerobic biodegradability. Further experimental investigations are thus needed in order to estimate the inhibitory effect of the MWF on methanogens in the anaerobic degradation process. These would employ the methods to reduce the inhibitory effect with the aim of enhancing the methane production process.

Chapter 4

Examining the Toxic Effects of the Metalworking Fluid on Methangenesis

4.1 Introduction

The sense of MWFs on exposed microorganisms, which is a toxicity effect, has often been highlighted in prior studies attempting to exploit biological systems for the disposal of MWFs, including aerobic and anaerobic routes (van der Gast et al., 2001; 2004; Perez et al., 2006). This is not surprising because MWFs are specifically formulated so that they are resistant to microbial colonisation and bio-deterioration whilst in use on machines. Most of previous studies regarding biological treatment of MWFs focused on aerobic treatment and few successful cases attempted anaerobic treatment processes because of the toxicity of MWFs (van der Gast et al., 2001; Jagadevan et al., 2011; 2012; Cheng et al., 2004; 2005).

The anaerobic degradation process of the waste MWF was investigated in this study because of its advantages, in comparison to the aerobic degradation process, such as its high loading rate, low energy cost, and the fact that it converts organic waste into bio-energy instead of just producing CO_2 emissions. Methanogens have been reported as the key to convert organic waste into methane; but they have been shown to be quite sensitive to unfavourable temperature, pH and toxicity environments (Dhaked et al., 2003;

2010; Patidar et al., 2005; Chen et al., 2007; .Hoj et al., 2008). However, it seems that there is relatively little known about the toxic effects of MWFs on different microorganisms in biological treatment processes, especially in anaerobic microbial communities that have the potential to be exploited in treatment processes, such as those used in the studies carried out by Kim et al. (1989; 1992a; 1994). This may be because of the complex compositions of different MWFs and the uncharacterised MWF samples that were used in these previous studies.

The inhibitory effects of the MWF on methane production rate were also found in the studies described in Chapter 3. In order to develop methods for overcoming the toxic effects and to enhance methane production, an investigation regarding the toxic effects of the MWF on methanogens was carried out employing molecular and culture approaches in the experiments described in this chapter.

To date, molecular profiling has been employed to characterise the diversity and community structure of microbial communities in biological wastewater treatment systems (Boone et al., 1993; Amann et al., 1995; Sekiguchi et al., 1999; Garcia et al., 2000; Scherr et al., 2012). The advent of some new molecular biological methods has greatly aided investigation into the biological disposal of MWFs. Two of these methods were employed in this study: fluorescence *in-situ* hybridisation (FISH) and denaturing gradient gel electrophoresis (DGGE).

In addition, a real-time analysis method based on a bioluminescence-based bacterial biosensor, *E.coli HB101_pUCD607_lux*, was developed in order to carry out the evaluation of the toxic effects of MWFs on the anaerobic degradation process.

The studies presented in this chapter aimed to investigate the toxic effects of the MWF sample on methanogens in the anaerobic degradation process of the MWF, and there were three major sections:

1. An investigation regarding the toxic effects of the MWF on the activity of methanogens;
2. An investigation regarding the effects of the MWF on the methanogen and the diversity of their community; and
3. The development of a real-time analysis method for evaluating the toxic effects of the MWF on its anaerobic degradation process.

4.2 Methods and Materials

4.2.1 Toxic Effects of the MWF on the Activity of Methanogens

This experiment was aimed at investigating the toxic effect of the MWF on the activity of the methanogens. Flocculent activated sludge and nutrient broth solution (see section 3.2.2 and 3.2.3 for details) were used in this experiment as the inoculum and main substrate. Resazurin (1mg/L, see section 3.2.4 for details) was added into the substrate as an anaerobic indicator.

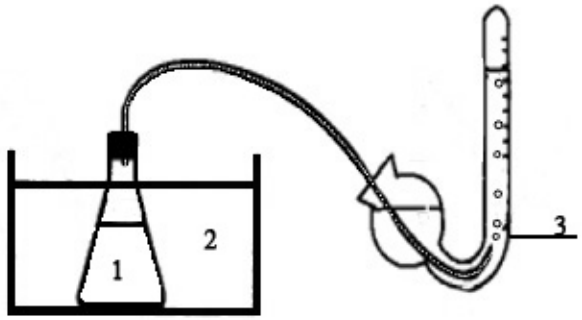

Figure 4.1: Design of the experimental system for investigating methane production rate (1: Water Bath. 2: 125 ml Flask. 3: Smith fermentation tube containing 11.2 %W/V KOH solution)

The set-up of the experimental system employed in this investigation is shown in Figure 4.1 above. A 125ml flask was used containing 90ml diluted nutrient broth solution (5,000mgCOD/L, pH 7.2), 1mg/L resazurin, 2g dry anaerobic activated sludge and a 10ml MWF sample at different concentrations (including 2,000mg/L, 5,000mg/L, 8,000mg/L and 10,000mg/L). The final pH value of the mixed substrate was adjusted to the same pH of the nutrient broth solution (7.2) to avoid any effect caused by different pH to the experiment, and this pH was also in the optimal pH range for methane

production investigated in section 3.3.2.2. The final COD concentrations of the MWF in the mixed samples were 200mg/L, 500mg/L, 800mg/L and 1000mg/L. The experimental system was flushed with N_2 gas for 10 minutes so as to remove O_2 until the resazurin became colourless. The volume of biogas production (including methane and hydrogen gas) was recorded every 30 minutes in the first eight hours and then every hour until no more methane was detected, and the concentration of methane in the biogas samples was measured using the GC-FID system (GC 2010 series; Shimadzu, UK). The flasks were shaken after sampling. The final pH values of the mixed substrates were measured, and a control sample group was established containing only 100ml nutrient broth solution.

The purpose of the experimental set-up (i.e. mixing the substrates with a large portion of growth medium and carrying out the experiment in a short period of time (40 hours)), was to reduce the immediate toxic effects of adding the MWF sample in terms of changing the inoculum itself, such as it altering the structure of the bacterial community during the experiment process.

4.2.2 Toxic Effects of the MWF on the Methanogen Community

Besides the toxic effects on the activity of methanogens, the toxicity of the MWF could also cause other effects in terms of changing the community composition of the methanogens, including displacement of more sensitive but essential populations (van der Gast et al., 2001; 2004; Perez et al., 2006; Cheng et al., 2004; 2005). The studies described in the following section are focused on investigating the effect of the MWF on the diversity and community structure of methanogens in the anaerobic sludge samples fed with the MWF sample for a long period of time in flask-scale reactor experiments

(see Chapter 3 for details) using two molecular biological methods: FISH and DGGE.

1. Samples for Genetic Analysis

The samples used for genetic analysis in this part of the study were the flocculent and granular sludge samples taken from the three replicate flask-scale reactors in the experiments described in sections 3.2.8 on day 1, day 10 and day 20 of the 20-day operation. The flocculent anaerobic activated sludge was collected from a sludge digester at Thames Water Oxford Sewage Works, and the granular sludge was collected from a full-scale Up-flow Anaerobic Sludge Blanket (UASB) reactor in a pharmaceutical wastewater treatment plant (For the details of the sludge samples, see section 3.2.2). The three replicate sludge samples of each type of anaerobic sludge were used for FISH and DGGE analysis.

2. Fluorescence *In-Situ* Hybridisation Analysis

FISH is a molecular biological technique utilising labelled DNA probes to detect specific genes on chromosomes. The denatured sample DNA was mixed with a fluorescently labelled probe, and the probe hybridises with the sample DNA at the target site as it re-anneals back into a double–stranded DNA. The probe signal can be examined by fluorescent microscopes and the sample DNA scored for the presence or absence of the signal (Green & Sambrook, 2012). The FISH probe experiments were carried out with assistance from Dr. Chun Liu and Mr. Xiaoxuan Chen from the School of Environmental Science and Engineering at the Hebei University of Science and Technology (Hebei, China). The FISH experimental procedure and details regarding the materials and

reagents used in the experiments are given in Appendix 2. The FISH probes (synthesised by Takara Ltd, Dalian, China) used in the genetic analysis of methanogens in are shown in Table 4.1 above.

Table 4.1: Methanogen FISH probes used in this study

Probes	Probe Sequence	Tag Type	References
16s rRNA	CATGCACCACCTCTCAGC	5′-HEX	Chun et al., 2010
mcrA	TTCATTGCRTAGTTWGGRTAGTT	5'-FITC	Friedrich et al., 2005; Juottonen et al., 2006
mtr	ATGTTYAAGTTYGAYAAGAAGCARG	5'-FITC	Chun et al., 2010
F_{420}	CACCAGACAGTCACAGTCCACTGG	5'-FITC	Song et al., 2010

16s rRNA probe is one of the universal probes targeting methangens, and mcrA, mtr and F_{420} are the important functional genes in methanogens encoding some unique coenzymes. mcrA gene is a "functional" marker gene coding a-subunit of methyl-coenzyme M reductase (MCR), the key enzyme of methanogenesis (Rastogi, et al., 2008). The process that methyl transfer from N^5-methyltetrahydromethanopterin to coenzyme M are dependent on sodium ions for methane formation, this reaction is catalysed by a Na-translocating membrane-associated multienzyme complex (MtrA); and mtr is the functional maker gene (Gottschalk & Thauer, 2001). The coenzyme F_{420} is an important enzyme involved in the redox reactions in methanogens, it exists in methanogens as an electron transport in the methane generation process and can be used to represent the activity of methanogens (Dolfing, & Mulder, 1985). These four probes were chosen in this study because they are the most popular ones in previous studies.

There were two parts to the FISH experiment. Firstly, the four methanogen FISH probes were employed with one flocculent sludge sample, and the results were analysed to select the optimal probe for subsequent FISH analysis. Secondly, the flocculent sludge samples and granular sludge samples taken from the flask-scale reactor experiments (described in sections 3.3.8) were analysed with the selected probe to investigate the effects of the MWF on the methanogen communities in the sludge samples. The FISH images were processed and analysed using Motic Fluo Software (Motic Incorporation Ltd. Hong Kong), and the relative abundances of the methanogens in sludge samples were calculated according to Equation 4.1 below:

$$Relative \text{ Abundance of Methanogens}(\%) = \frac{Area\ of\ the\ sample\ image\ hybirdised\ with\ methanogen\ probe}{Whole\ area\ of\ the\ sample\ image} \times 100\% \quad \text{(Eq. 4.1)}$$

3. Denaturing Gradient Gel Electrophoresis Analysis

DGGE is a molecular biology approach used for the monitoring of microbial populations. It is a fingerprinting methodology that employs chemical gradients to denature the samples as it moves across acrylamide gels (Green & Sambrook, 2012). It has been widely applied to nucleic acids for investigating interactions among microorganisms and their environment (Scherr et al., 2012; Cheng et al., 2008). The DGGE work was carried out with assistance from Mr Xinkun Ren from the Department of Inorganic Chemistry at the University of Oxford. For details of the DGGE experimental protocol, see Appendix 3, while the procedures of the genomic deoxyribonucleic acid (DNA) extraction and the

Polymerase Chain Reaction (PCR) are described in Appendix 4 and Appendix 5, respectively. The 16S rRNA was enzymatically amplified by PCR using methanogen primers with a GC clamp (synthesised by Sigma-Aldrich, UK), including BSF338 (5′-ACT CCT ACG GGA GGC AGC AG-3′) and BSR534 (5′-ATT ACC GCG GCT GCT GGC-3′) (GC clamp:5′-CGC CCG CCG CGC CCC GCG CCC GTC CCGCCG CCC CCG CCC G-3′) (Cheng et al., 2008).

In addition, the electric voltage for carrying out DGGE was optimised at the beginning of this experiment. Two standard gels were run with two electric voltages of 60V and 120V. The results are shown in Figure 4.2 below. It can be seen that more clear bands, standing for methanogen strains, appeared in the gel run at 60V for 12 hours; thus, 60V was selected for operating the DGGE in this study.

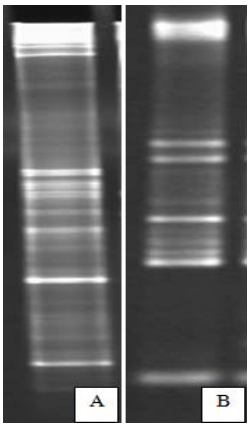

Figure 4.2: The results of optimising the experimental electric voltage for PCR-DGGE at 60V for 12 hours (A) and at 120V for six hours (B).

4.2.3 Toxicity Analysis of the MWF

This study aimed to develop an approach for the real-time analysis to determine the bio-toxicity of the MWF samples and the treated samples.

The potential toxic effects of the MWF on the anaerobic degradation process was reflected in the marked decrease of the COD removal efficiency and methane production in the flask-scale experiments (described in Chapter 3) and the changes to the methanogen community in the anaerobic sludge experimented on with the MWF (described in sections 4.3.1 and 4.3.2). However, these individual indicators when employed alone are not capable of characterising the overall toxicity of the MWF on an anaerobic degradation system in subsequent studies; and the experimental methods are time-consuming. Therefore, a bacterial biosensor was employed for subsequent studies to evaluate the toxic effects of MWFs on the anaerobic degradation process. This toxicity analysis approach is based on a bioluminescence-based bacteria biosensor – *E.coli HB101_pUCD607_lux* (Mwinyihija, 2011), and it is suitable for real-time analysis. The toxicity test method and procedure were developed with two of my colleagues, Andrea Chan and Meg Uapipatanakul, from the Department of Engineering Science at the University of Oxford.

Based on the toxicity analysis method, the toxicity value of MWFs employed in this study was defined as the **Relative Toxicity** (Eq. 4.2)

$$\text{Relative Toxicity} = \frac{L_{Blank} - L}{L_{Blank}} \times 100\% \qquad \text{(Eq. 4.2)}$$

L: luminescence reading of test sample.

L_{Blank}: luminescence reading of blank/control sample.

Briefly, there are four stages in the toxicity analysis procedure: (1) determination of the mid-exponential phase of the biosensor growth; (2) preparation of the freeze-dried stock of the biosensor; (3) initial toxicity test procedure; and (4) optimisation of the exposure time in the toxicity test procedure. The details of the four stages are given below.

1. Determination of the Mid-Exponential Phase

The mid-exponential phase of the biosensor's growth is the optimal phase for carrying out the toxicity analysis, because the *E.coli HB101_pUCD607_lux* has relatively greater luminescence intensity in this growth phase. It can be identified by plotting the growth curve of the biosensor. The details of the experimental procedure are given as follows:

1) Prepare culture medium. The culture medium is based on lysogeny broth (LB broth, Sigma-Aldrich, UK), and the other ingredients added are: 10g/L Tryptone (OXOID), 5g/L yeast extract (Fisher Scientific), 5g/L sodium chloride (Fisher Scientific), 1g/L glucose and 25ug/L ampicillin (Sigma-Aldrich, UK). Glucose and ampicillin are dissolved in deionised water as a stock solution, and added into the autoclaved culture medium.
2) Incubate *E.coli HB101_pUCD607_ lux* in 5ml sterile LB Broth as inoculum, at 35℃, 120rpm, overnight in a shaking incubator (Innova 44, New Brunswick Scientific).
3) Transfer the *E.coli HB101_pUCD607_lux* inoculum into the 100ml sterile LB broth and continue the incubation in order to produce the growth curve and luminescence curve.

4) Record the optical density (OD) and luminescence of the culture every 30 minutes (Shimadzu UV-1800 UV spectrophotometer, BioTek Synergy HT). The result is shown in Figure 4.3 below.

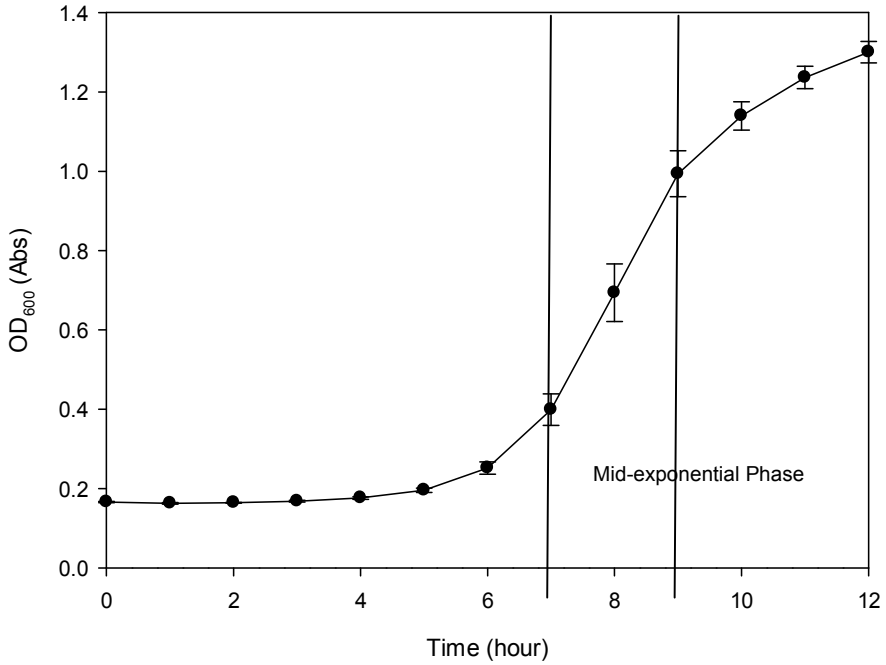

Figure 4.3: Growth curve of *E.coli HB101_pUCD607_lux* in LB broth with ampicillin and the mid-exponential phase (n=3, Error Bar: SD).

It can be seen from Figure 4.3 that, the mid-exponential phase of *E.coli HB101_pUCD607_ lux* in LB broth came at around nine hours. Based on this, the freeze-dried stock of *E.coli HB101_pUCD607_ lux* was prepared for toxicity analysis when cells were in mid-exponential phase.

2. Freeze-dried Stock Preparation Procedure of the Biosensor

1) First, add 5 ml of an overnight culture of *E.coli HB101_pUCD607_ lux* to 100ml of sterile LB broth (Sigma-Aldrich, UK) with 1g/L glucose and 50µg/ml ampicillin.

2) Incubate the culture in a shaking incubator at 37℃ at 150rpm for nine hours until the culture reaches the mi-exponential growth phase.
3) Place the culture on ice immediately, and then centrifuge it at 3,000 rpm at 4℃ for 20 minutes.
4) Re-suspend the cell pellet in sterile *Mist dessicans* (Vserum:VLB= 3:1, 10g/L glucose added in LB broth; Heckly, 1961).
5) Dispense 2ml of the suspension into each sterile freeze-drying vial (Wheaton, vial lyophilisation glass clear 5ml), partially seal them with rubber stoppers, and freeze at -80℃ overnight.
6) Lyophilise the frozen cells in an LTE cryopreservation unit (mini-Lyotrap, LTE) for 24 hours at -55℃.

3. Toxicity Test Procedure

The experimental procedure of the toxicity test is shown as follows:

1) Load 180ul of each sample into a 96-vial plate (Nuncbrand).
2) Re-suspend the *E.coli HB101_pUCD607_lux* freeze-dried stock in 2ml distilled water, and leave the culture at 25℃ for two hours.
3) Wash the cells with phosphate buffer solution (PBS, 0.01M, pH 7.4; Sigma-Aldrich, UK).
4) Inoculate 20ul of the culture into each well of the 96-vial plate.
5) Measure and record the relative light units after 15 minutes of exposure using the plate reader (BioTek Synergy HT). The determination of the exposure time is introduced in the following section.

4. Optimisation of the Exposure Time in the Toxicity Analysis Procedure

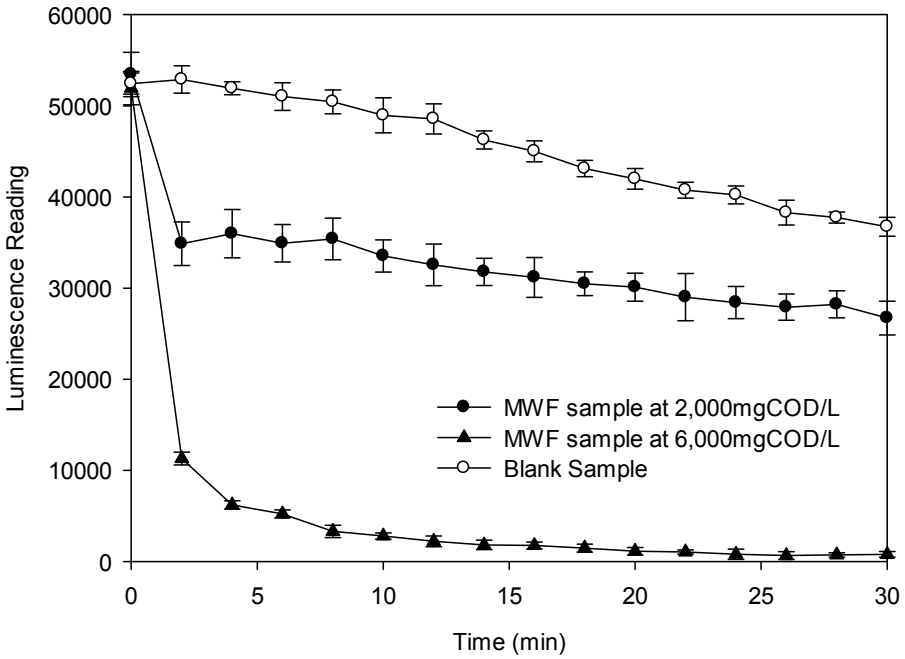

Figure 4.4: Changes in the relative toxicity of a blank sample without MWF and two diluted MWF samples using *E.coli HB101_pUCD607_lux* during the exposure time of 30 minutes (n=3, Error Bar: SD).

The exposure time for the toxicity analysis of the MWF was optimised with a blank sample (only LB broth) and two diluted MWF samples at 2,000mgCOD/L and 6,000 mgCOD/L. The results are shown in Figure 4.4.

It can be seen that the concentration of the MWF sample at 6,000 mgCOD/L was too high to carry out the toxicity analysis because the reduction in the luminescence readings was too rapid. of all three samples stabilised around 15 minutes of exposure. Thus, 15 minutes was selected as the exposure time in the toxicity analysis method used in this study.

In addition, because the toxicity analysis method was based on a microorganism and its luminescence activity, the testing process may be affected by the changing pH value of

the samples. Thus, an experiment aimed at investigating the effect of sample pH value on the evaluation of the toxicity was carried out. PBS samples of the same concentration over a range of pHs were prepared, including 1, 2, 4, 5, 6, 6.5, 7, 8, 9; and their relative toxicity values determined employing the method described in section 4.2.5. The PBS samples were mixed with the biosensor cultures, and final pH values of the mixtures were 1.16, 1.97, 3.93, 4.97, 6.12, 6.55, 7.04, 7.91, and 9.07. The luminescence readings of the samples were determined with the plate reader (BioTek Synergy HT). According to the standard curve and previous results on the pH value, pH 7 was selected for carrying out the tests of relative toxicity.

5. Toxicity Analysis of the Raw and Treated MWF Samples

The aim of this study is to evaluate the relative toxicity of the MWF at different concentrations and its changes after the anaerobic treatment with activated sludge.

The raw MWF samples, which were diluted to 0.5%, 1%, 2%, 2.5%, 5% and 10% v/v were prepared in triplicate, and their pH values were adjusted to 7 with 0.1M HCl. The relative toxicity values of the samples were determined and calculated according to the method introduced above. A blank sample was prepared without the MWF sample.

Then, 30 replicates MWF samples inoculated with flocculent sludge were prepared with in order to investigate the changes in the MWF's relative toxicity during the anaerobic treatment process. In each sample, 15ml of the MWF sample (5,000mgCOD/L, pH 7) was kept in a sealed culture tube (total volume: 25ml) with 0.5g dry anaerobic flocculent sludge and 1mg/L resazurin. The culture tubes were flushed with N_2 gas until the

resazurin become colourless; they were then sealed with rubber caps. They were kept in a shaking incubator at 35℃ for two weeks. In each sampling time, three replicates were used to determine the total COD reduction rate, the cumulative methane production rate and the relative toxicity of the treated MWF samples.

4.3 Results and Discussion

4.3.1 Toxic Effects of the MWF on the Activity of Methanogens

The results of the flask-scale bottle reactor experiments described in Chapter 3 revealed that adding the MWF to the sample as a substrate caused significant inhibitory effects on the anaerobic bacteria communities in terms of reducing the overall COD reduction efficiency and diminishing the methane production rate. This experiment aimed to investigate the inhibitory effects of the MWF on the activity of the methanogens, which play a pivotal role in the methanogenesis of the anaerobic degradation process.

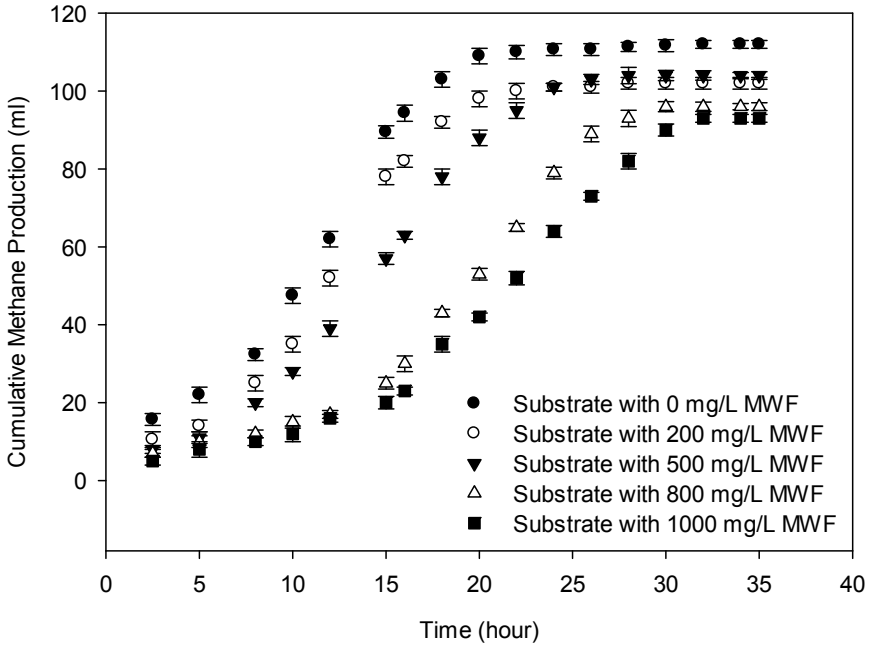

Figure 4.5: Changes in cumulative volume of methane production from mixed substrates with different concentrations of the MWF (n=3, Error Bar: SD).

Figure 4.5 shows the changes in the cumulative volume of the methane produced from the mixed substrates mixed with the MWF samples at different concentrations. There was no significant pH change in the substrates during the experimental period. It can be seen

from Figure 4.5 that, the overall volume of methane production decreased with increasing concentration of the MWF in the substrate. This finding confirmed the inhibitory effect of the MWF on the overall activity of methanogens and the biodegradability of the MWF sample was much lower than the nutrient broth substrate. This finding also suggests that the purpose of the experiment set-up was fulfilled. The small reductions in the overall volume of methane production should mainly be caused by the different amounts of MWF sample added in the mixed substrates. Moreover, the methane production rates decreased with the increasing concentration of the MWF sample.

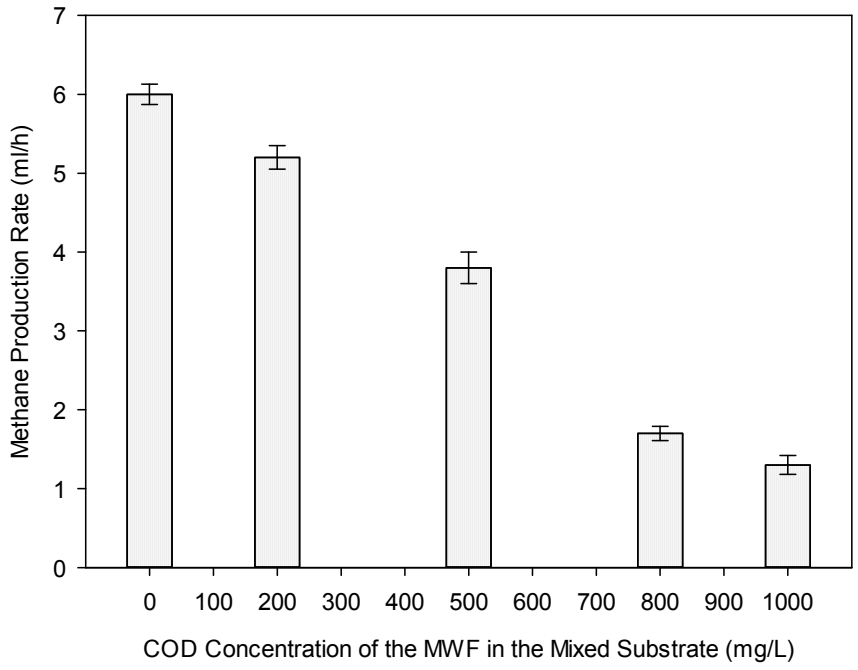

Figure 4.6: Methane production rates at different concentrations of the MWF at 15 hours (n=3, Error Bar: SD).

Figure 4.6 above represents the methane production rates from the mixed substrates with different concentrations of the MWF in the first 15 hours of the operation. It can be seen from Figure 4.6 that, the methane production rate at the first 15 hours declined from 6 ml/h to 1.3ml/h with the increasing concentration of the MWF in the mixed substrates.

This clearly suggests the existence of a strong inhibitory effect on the part of the MWF in terms of the activity of the methanogens. Figure 4.5 shows that, with the increasing concentration of the MWF sample, more time was required for the activity to recover from the shock effect. Compared with the blank control group, the group fed with the mixed substrate containing the 1,000mg/L MWF sample required almost double the time to recover from the inhibitory effect.

4.3.2 Toxic Effects of the MWF on the Methanogen Community

1. Fluorescence *In-Situ* Hybridisation Analysis

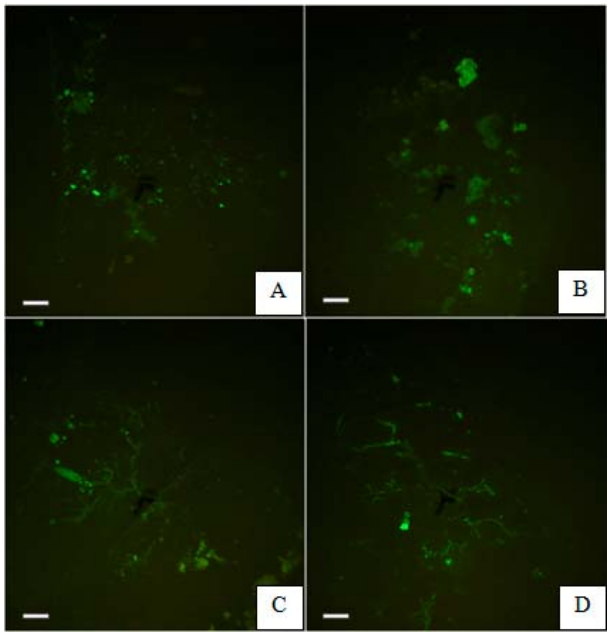

Figure 4.7: Fluorescent microscope images of the flocculent sludge tested with the four types of methanogen FISH probes, the bright colours indicate the existence of methanogens (A:16s rRNA probe; B: mcrA gene probe; C: mtr gene probe; D: F_{420} gene probe, scale bar=200μm).

As was introduced in section 4.2.2.2, the results of the FISH analysis can demonstrate the changes in the proportion of a specific species – methanogens in this study – in a whole microorganism community. Figure 4.7 above shows the fluorescent microscope images

of the flocculent sludge sample tested with the four types of methanogen FISH probes. The green luminous spots in the images represent the existence of methanogens in the sludge sample. In other words, this provides further evidence that the anaerobic disposal of the MWF is a viable option with added potential benefit of generating bio-energy in the form of methane from the wastes.

In order to select the optimal probe for the further FISH analysis, the four methanogen FISH probes were used to analyse the flocculent sludge samples taken from the flask-scale reactor experiments on day 0, day 10 and day 20 of the 20-day operation. The results were compared in order to select a FISH probe for further studies. The results are given in Tables 4.2. The fluorescent microscope images of the FISH experiment were processed with Motic Fluo Software (Motic Incorporation Ltd. Hong Kong). The relative abundance of methanogens in the samples was calculated according to Equation. 4.1.

Table 4.2: Relative abundances (%) of methanogens analysed with the methanogen probes in the flocculent sludge samples fed with the MWF at 0 days, 10 days and 20 days.

Sampling Day / Probes	Day 0	Day 10	Day 20
16s rRNA	39.52±2.35	30.21±2.11	34.64±1.85
mcrA	26.92±2.34	20.23±2.82	22.24±2.40
mtr	39.26±2.31	30.71±2.15	36.54±2.54
F_{420}	35.53±2.17	28.21±3.04	30.56±2.32

It can be seen from the results presented Tables 4.2 that the relative methanogen abundances of the same sludge sample were different for each methanogen FISH probe employed. The average relative methanogen abundances of the flocculent sludge sample

analysed with the four FISH probes are summarised in Figure 4.8 below for comparison. In addition, the results were analysed using ANOVA-test given in Table 4.3 below. In this study, it was considered a statistically significant difference when the p-value of the t-Test was at 0.05 or below.

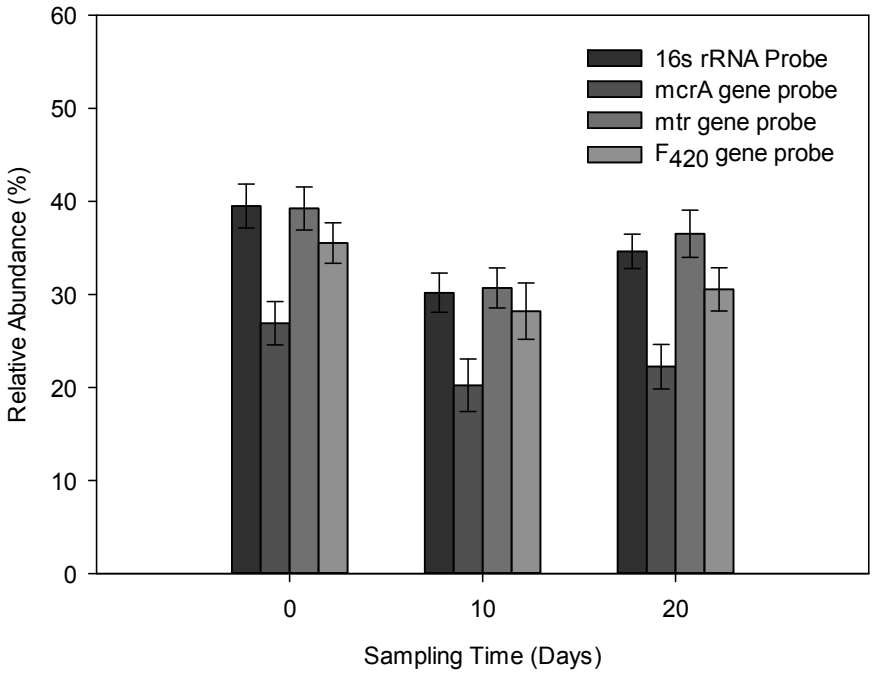

Figure 4.8: Average relative methanogen abundances of the flocculent sludge samples taken over 20-day operation with the four FISH probes.

Table 4. 3: Results of ANOVA test of the relative abundances (%) of methanogens analysed with the methanogen probes

Source of Variation	DF	SS	MS	F	P
16s rRNA					
Between Groups	2	130.11	65.06	14.568	0.005
Residual	6	26.79	4.47		
Total	8	156.90			
mcrA					
Between Groups	2	70.70	35.35	5.53	0.044
Residual	6	38.38	6.40		
Total	8	109.08			
mtr					
Between Groups	2	114.49	57.25	10.47	0.011
Residual	6	32.82	5.47		
Total	8	147.31			
F_{420}					
Between Groups	2	83.81	41.90	6.50	0.031
Residual	6	38.67	6.44		
Total	8	122.48			

The ANOVA results show that the data of the relative methanogen abundances of the sludge samples taken at the three points were statistically significant different. However, as shown in Figure 4.12, the changes at the three points over the 20-day operation did not exceed 25%. This may suggest a good buffer capability on the part of the flocculent sludge in regard to a toxic environment. In addition, the FISH probe based on 16s rRNA was selected for this study; because the p-value of the ANOVA-test is 0.005. The FISH images of the flocculent sludge samples analysed with the 16s rRNA probe are given in Figure 4.9 below.

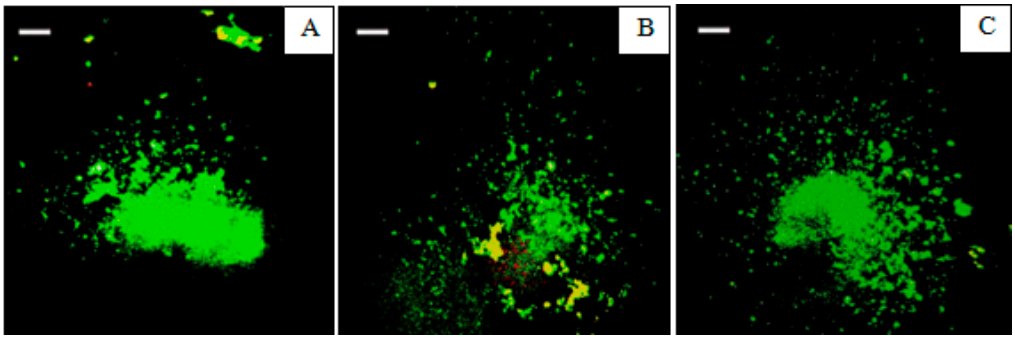

Figure 4.9: Processed fluorescent microscope images of the flocculent sludge samples analysed with the 16s rRNA methanogen FISH probe (A: raw sludge sample, B: sludge sample fed with the MWF for 10 days, C: sludge sample fed with the MWF for 20 days; the brightly green colours indicate the existence of methanogens, scale bar=100μm).

Subsequently, the 16s rRNA methanogen probe was also used to analyse the granular sludge samples fed with the MWF sample in the flask-scale reactor experiments over the 20-day operation. The results are presented in Figure 4.10 and Table 4.4 below.

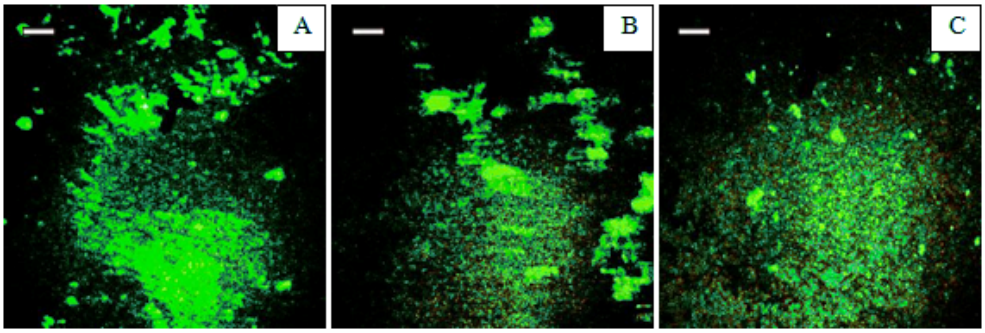

Figure 4.10: Processed fluorescent microscope images of the granular sludge samples analysed with the 16s rRNA methanogen FISH probe (A: raw sludge sample, B: sludge sample fed with the MWF for 10 days, C: sludge sample fed with the MWF for 20 days; the brightly green colours indicate the existence of methanogens, scale Bar=100μm).

Table 4.4: Relative abundances of methanogens analysed with the 16s rRNA methanogen probe in the granular sludge samples fed with the MWF at 0 days, 10 days and 20 days.

Sludge Sample	Day 0	Day 10	Day 20
Relative Abundance (%)	54.31±3.15	42.08±2.44	46.14±3.21

Compared with the results of the flocculent and granular sludge samples with the 16s rRNA methanogen probe (shown in Table 4.2 and Table 4.4), it can be seen that the portion of methanogens in the granular sludge was greater. This may explain the greater methane production rate of the granular sludge in comparison to the flocculent sludge in the flask-scale reactor experiments. It also suggests the excellent potential in terms of undertaking anaerobic degradation of the MWF whilst converting it to bio-energy in the form of methane.

The FISH image analysis is convenient method for the detection of methanogens in this study. However, it also has inevitable limitations in terms of accuracy, such as the fluorescent signal may be falsely enhanced by multi-layered cells. The results obtained from the studies employing FISH probes indicate that there was an unapparent change in the overall biomass of methanogens in the flocculent and granular sludge over the 20-day operation, but this was investigated further by DGGE.

2. Denaturing Gradient Gel Electrophoresis Analysis

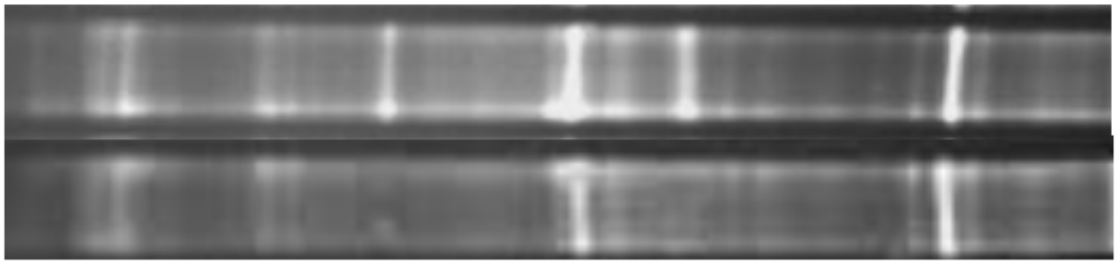

Figure 4.11: Changes in genotypic profiling by DGGE analysis of the flocculent sludge used in the flask-scale reactor experiments over the 20-day operation with the MWF (A: raw sludge sample, B: sludge sample fed with the MWF for 20 days).

Figure 4.11 shows the changes in the genotypic profiling of the flocculent sludge over the 20-day operation with the MWF sample in the flask-scale reactor experiments described

in Chapter 3. Each band is taken to correspond to individual methanogen strain. It can be seen that, there was a significant change in the community structure of methanogens in the sludge sample, with some methanogen bands having disappeared during the 20-day operation fed with the MWF sample. This suggests that the methanogen species – represented by the bands that had disappeared – were more sensitive to the toxicity effect of the MWF. Moreover, this result also illustrates that some of the methanogen species may have relatively greater activity in the MWF, which suggests there is a good level of feasibility for employing this particular species to enhance methane production in MWFs' anaerobic degradation process.

To sum up, the results described in sections 4.4.1 and 4.4.2 suggest that the toxicity of the MWF has significant effects on the overall activity of methanogen species and alteredthe structure of methanogen communities. The potential toxic effect of the MWF was examined in more details by employing a bacterial biosensor.

4.3.3 Toxicity Analysis Results of the MWF

A simple and real-time measurement approach of the toxicity of the MWF was developed for subsequent studies with a bioluminescence-based bacterial biosensor, *E.coli HB101_pUCD607_ lux* (Mwinyihija, 2011).

1. Effect of pH on the Relative Toxicity Analysis Method

The toxicity analysis method based on a bacterial biosensor is known to be affected by certain environmental factors, such as the temperature and pH of the test samples. The effect of a range of pH values on the biosensor reading was investigated. The results

illustrate a standard curve of the relationship between sample pH value and the luminescence reading determined for the relative toxicity analysis, as shown in Figure 4.12.

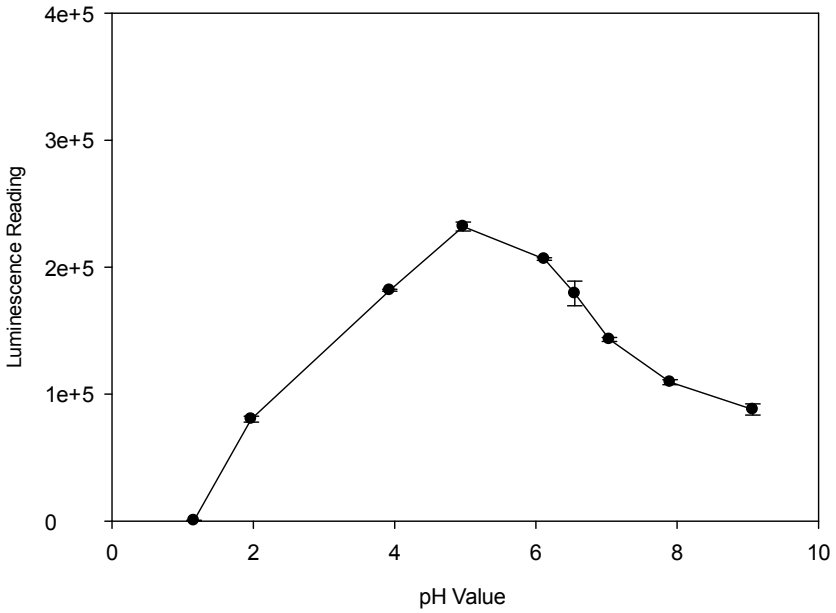

Figure 4.12: Relationship between the sample pH value and the luminescence reading determined for the relative toxicity analysis with *E.coli HB101_pUCD607_lux* (n=3, Error Bar: SD).

It can be seen from Figure 4.12 that, the pH of the sample caused a significant difference in the bio-luminescence reading recorded, which can in turn cause a significant error in the calculation of the relative toxicity. Thus, the optimal test condition would be to carry out the analysis with the samples at a standard pH, which was selected at pH 7 in this study.

2. Relative Toxicity of the Raw Metalworking Fluid Sample

Figure 4.13 below shows the results of employing the relative toxicity analysis developed in this study on the raw MWF samples at different concentrations and the same pH value.

The figure shows that, the values of the relative toxicity of the MWF samples at 2,000mgCOD/L and 5,000mgCOD/L (pH 7) were 76.7% and 94.8% respectively.

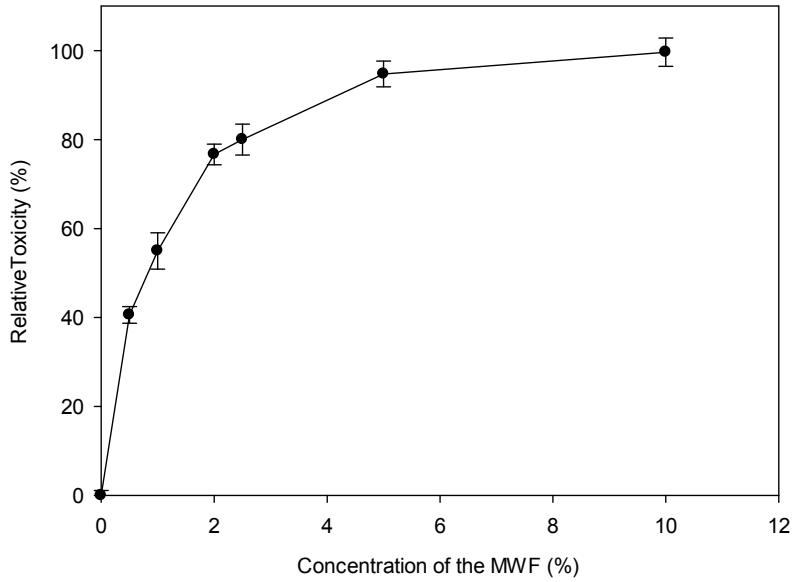

Figure 4.13: The relative toxicity of the MWF samples at different concentrations (n=3, Error Bar: SD).

It can be seen from Figure 4.13 that the relative toxicity reading of the MWF diluted to 10% of its raw concentration was around 100%. It means this relative toxicity analysis reached it upper limit of measurement, and it was not suitable for the toxicity analysis of the MWF samples where the COD concentration was higher than 10% (10,000mgCOD/L). All the relative toxicity analyses in this study were thus carried out in the effective test range.

3. Changes in the Relative Toxicity of the MWF Treated with Activated Sludge

In the experiment, the changes in COD reduction, methane production rate and relative toxicity of the MWF sample were investigated in order to identify their relationships; and, subsequently, to investigate the feasibility of employing the toxicity analytical method in

this study. It was found the majority of the COD reduction, methane production and reduction of the relative toxicity of the MWF happened in the first eight days of the two-week experiment. Thus, the data obtained in the eight days were processed to identify the relationship between the total COD reduction rate, the daily methane production rate and the relative toxicity of the MWF sample. The final results are summarised in Figure 4.14.

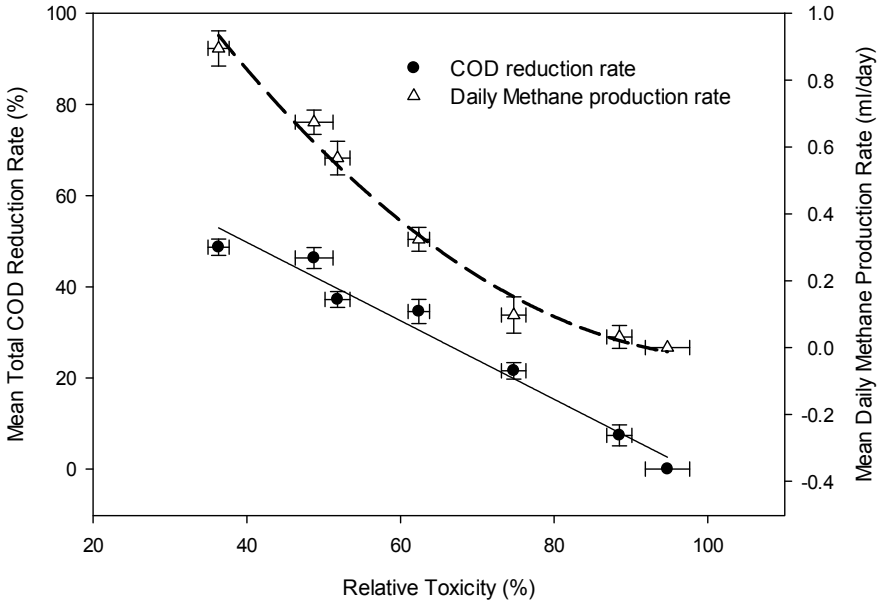

Figure 4.14: Relationship between the mean total COD reduction, the mean daily methane production rate and the relative toxicity in regard to the treatment of the MWF sample (5,000mgCOD/L pH 7) with flocculent sludge (n=3, Error Bar: SD, t=8d).

It can be seen from Figure 4.14 that the COD reduction rate of the experimental system inoculated with flocculent sludge decreased from around 55% to 0% with increasing relative toxicity from around 35% to 95%, while the methane product rate reduced from around 0.9ml/day to 0ml/day. Moreover, the result represented in Figure 4.14 also show there was a linear relationship between the total COD reduction and the relative toxicity of the treated MWF sample, as well as a good quadratic relationship between the daily methane production rate and the relative toxicity. Furthermore, the correlation

coefficients were 0.9842 (R^2=0.9686) and 0.9938 (R^2=0.9877). Thus, these results suggest that the relative toxicity analysis method is a feasible method with good accuracy in terms of its application in this study.

4.4 Conclusions

The findings of the studies described in Chapter 3 suggest that, in general, the MWF had remarkable inhibitory effects on the anaerobic degradation process in terms of both the COD reduction efficiency and the methane production rate. However, it had a much greater effect in regards to the reduction of methane production rate. The major aim of the studies carried out in this Chapter was to investigate the toxic effects of the MWF on methanogens employing molecular and culture approaches.

Overall, the results of the studies regarding methanogens revealed the significant toxic effects of the MWF on the activity and biodiversity of methanogens in the anaerobic degradation process of the MWF in regard to reducing the activity and altering the community structure of methanogens. The following three major conclusions and further discussion can be drawn from the studies outlined in this chapter:

I. The MWF had a significant inhibitory effect on the activity of methanogens, and this increased as the concentration of the MWF also increased. An obvious shock effect on the operation of bio-reactors was detected. The FISH and DGGE analyses revealed that the MWF caused some changes to the methanogen diversity in anaerobic sludge over the operation period of the flask-scale reactors. It seems that the MWF was very toxic to specific populations within the

methanogen community, which suggests the possibility of employing those methanogen species with less sensitivity to the MWF so as to improve the methane production rate in an anaerobic degradation process of the MWF. Thus, an isolation approach of methanogens indigenous and adapted to existence in MWFs was developed and employed in the studies described in following Chapter (Chapter 5).

II. The results of the FISH and DGGE analyses of the anaerobic sludge used to treat the MWF confirmed the existence of methanogens and that the anaerobic degradation of the MWF resulted in produced of methane, and the findings are consistent with those of Kim et al. (1989; 1990; 1992) who found the biodegraded COD in a MWF was converted to methane in a granular activated carbon (GAC) fluidised-bed reactor. The comparison of the FISH results between the flocculent and the granular sludge shows that the amount of methanogen biomass in the granular sludge was greater than that in the flocculent sludge. This could be one of the major reasons why the methane production was greater from the reactors inoculated with granular sludge than those inoculated with flocculent sludge seen in the flask-scale reactor experiments described in Chapter 3. It can thus be suggested that increasing the biomass of methanogens in an anaerobic degradation system could be a good way of enhancing methane production from waste MWFs. However, considering the significant toxic effect of the MWF on methanogens concluded above, without reducing the toxicity of waste MWFs, only increasing the biomass of methanogens in an anaerobic degradation system would not be a feasible or practicable solution to enhance the anaerobic degradation rate of spent

MWFs and methane production rate.

III. The relative toxicity of the MWF decreased in an anaerobic sludge experimental system. According to findings in previous studies regarding the functions of COD-reducing bacteria and methanogens (Bryant, 1979; Appels et al., 2008), COD-reducing bacteria reduced the toxicity of the MWF by degrading the toxic compounds, which resulted in the chemical conditions in the MWF (pH and bio-toxicity) being changed. Hence, it could conceivably be hypothesised that it should be a preferred toxicity-overcoming solution to isolate and exploit the COD reducing bacteria which can efficiently degrade the toxic compounds in MWFs.

To sum up, the findings of the studies in Chapter 3 and this Chapter have important implications for developing an approach to overcome the toxic effect of MWFs on anaerobic degradation process by employing specific population that have adapted to the toxic environment of the MWFs. This is strong supporting evidence to support the proposition that exploiting specific COD-reducing bacteria in the treatment of the MWF could be an efficient method to overcome its toxicity so enabling subsequent biotreatment. The rationale then was to isolate and cultivate the anaerobic bacteria species colonising spent MWFs and then re-inoculate them back into the treatment system. The results regarding the relative toxicity analysis shown in Figure 4.17 also suggest that the methane production rate of methanogens could be increased by overcoming the toxicity of MWFs in this way. Thus, this approach was aimed at overcoming the toxic effects of MWFs so enabling stimulation of methane generation.

However, investigations into the performance of COD-reducing bacteria on reducing the

relative toxicity of the MWF can not be effective employing a mixed culture which have indigenous community of methanogens, such as occurs activated sludge. So it could only can be done by using pure cultures of COD-reducing bacteria acclimated in the toxic condition of the MWF.

Chapter 5

Overcoming the Toxic Effects of the Metalworking Fluid on the Anaerobic Degradation Process

5.1 Introduction

Among the studies on the disposal of spent MWFs, there have been comparatively few reported studies investigated the anaerobic degradation of MWFs (Cheng et al., 2005) as a treatment method. Three reasons could explain this lack of attention: 1) the toxicity of MWFs; 2) the sensitivity of anaerobic bacteria to toxic environments; and 3) the difficulties in isolating, cultivating and exploiting anaerobic bacteria.

The great toxicity of MWFs is commonly highlighted in previous studies regarding the anaerobic disposal of operationally exhausted MWFs (Kim et al., 1989; 1990; 1992, 1992a; Cheng et al., 2004, 2005; Perez et al., 2006). In this study, the toxic effects of a typical semi-synthetic MWF (RELUBRO 800) on the anaerobic degradation processes and methanogenic activity were confirmed in the experiments described in Chapter 3 and Chapter 4. The sensitivity of anaerobic bacteria to the toxic environment in RELUBRO MWF resulted in inhibition in terms of degradation efficiency of COD reduction and methane production rates.

In contrast, the literature review revealed that prior studies of the microbiology of spent and in-use MWFs demonstrate that many aerobic bacteria species are able to degrade

MWFs and adapt to their toxicity (Mattsby-Baktzer et al., 1989; van der Gast et al., 2001; Lodders et al., 2012; Saha et al., 2012). In Chapter 3 and Chapter 4, the most striking finding was that many anaerobic bacteria, including COD-reducing bacteria and methanogens, could survive in the toxic environment of RELUBRO MWF while degrading the biodegradable compounds and generating methane. Therefore, these findings suggested a promise in regards to overcoming the toxicity of the RELUBRO MWF by exploiting specific anaerobic bacteria species, which had previously adapted to the toxic conditions of MWFs, in the treatment process.

The rationale was to isolate and cultivate the anaerobic bacteria species colonising spent MWFs and then re-inoculate them back into the treatment system. However, this approach is undoubtedly challenging, not least because the isolation and cultivation of obligate anaerobes, such as methanogens, requires strict anaerobic experimental conditions. In addition, without the aid of recent bio-molecular analytical techniques, less than 1% of the anaerobic bacteria species present in the environment can be isolated and identified using traditional culture-based techniques (Amann et al., 1995). Thus, the approach was to exploit recently developed bio-molecular analytical techniques to identify the presence of target populations and then employ traditional culture-based techniques for isolating appropriate anaerobic bacteria indigenous to spent MWFs.

In this study, anaerobic bacteria were divided into two main categories according to their functional roles in the anaerobic degradation processes: 1) COD-reducing bacteria; and 2) methanogens. The COD reducers consisted of hydrolytic, acidogenic and acetogenic bacteria that degrade the organic compounds within the MWFs into small-molecule

fractions, which are subsequently assimilated and converted to biogas by the methanogens.

In the studies described in this chapter, some anaerobic bacteria colonising spent MWFs were isolated and exploited by subsequently re-inoculating them into RELUBRO MWF. This approach was aimed at overcoming the toxic effects of RELUBRO MWF and stimulating the generation of methane. The study described in this chapter was broken down into five sections as follows:

1. The development of the experimental methods for isolating anaerobic bacteria from spent MWFs;
2. The selection of the exploitable isolates for subsequent studies, according to their activities in RELUBRO MWF;
3. The identification of the exploitable isolates by genetic sequencing;
4. The investigation of the optimal growth conditions for the exploitable isolates; and
5. Overcoming the toxic effects of RELUBRO MWF by assembling and employing a mixed consortium using the exploitable isolates.

5.2 Isolation of Anaerobic Bacteria from Spent MWFs

The first step of the studies in this chapter describes the process of obtaining the exploitable isolates from spent MWFs, and is the key for all subsequent studies using the isolates, such as overcoming the toxicity of RELUBRO MWF. The isolation approach developed in this study is outlined in the following section.

5.2.1 Methods and Materials

The streak plate method (Sharma, 2007, pp. 149–173; Becker et al., 1996) was employed to isolate anaerobic bacteria from spent MWFs, and it was carried out with an anaerobic indicator, anaerobic jar, anaerobic atmosphere bag, and redox potential (Eh) reducers. The major steps of the bacteria-isolation approach are: i) isolation; ii) purification; and iii) cultivation (see Figure 5.1).

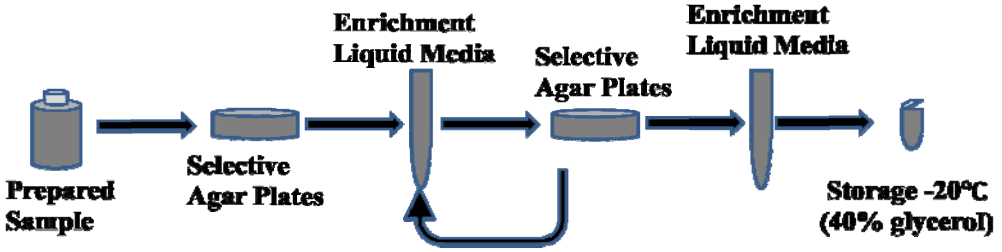

Figure 5.1: Bacteria-isolation process employed in this study for isolating anaerobic bacteria from spent MWF samples

Twenty different spent MWF samples that were spatially and temporally separated, provided by Microbial Solutions Ltd (a company specialising in disposal of waste MWFs), were used as sources for isolating anaerobic bacteria. These samples were as follows: RELUBRO 800, TR2000C, PAG 7, Hysol 113 Coll, AETC 3380, Shipman 06-130, Rolls Royce 7/6/10, F22 system, T3 Cell3 Oil, EM400/WP45, Hongh Tong Mix

Tank, STA-PUT G68, Hysoh Mix, DK331, EM400/WP45, SISTEMA F20, Bridgeport 10-355, B205LB, DK014S-24, and Microcut 371. All the samples had been kept in sealed bottles for several months before they were used in the bacteria-isolation procedures. However, the chemical compositions and formulation of the MWF waste streams were not available since they are commercially guarded information.

1. Establishment of Anaerobic Experimental Conditions

Resazurin (1mg/L, Acros Organics) was used as an anaerobic indicator added to the broth and agar media used in this study (for details about resazurin, see section 3.3.4). Then, two Eh reducers were employed for removing the oxygen existing in the media: Na_2S and L-Cysteine (Hungate, 1950; Balch et al. 1976; Fukushima et al., 2002). The anaerobic agar plates were incubated in an anaerobic jar (AnaeroGen Oxoid) employing an anaerobic atmosphere kit (AnaeroGen, Oxoid). Figure 5.2 shows the resazurin colour changes in the broth medium and agar plates.

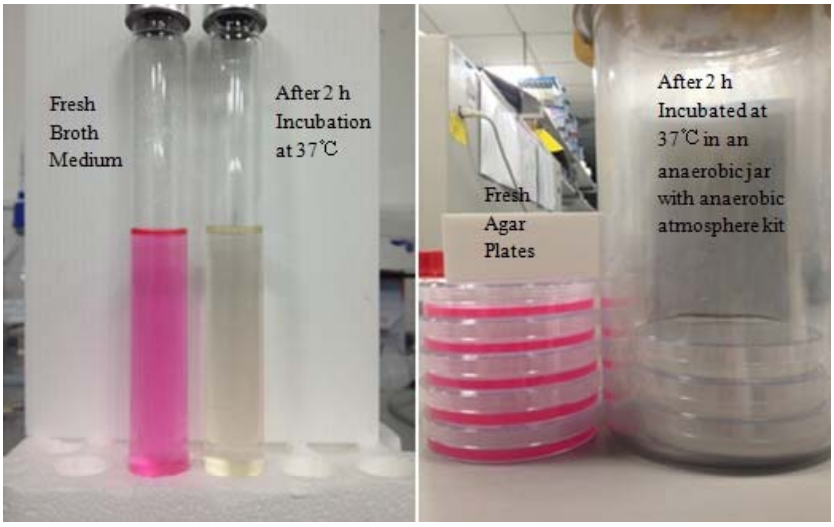

Figure 5.2: Shows colour changes in the broth medium and agar plates with 1 mg/L resazurin added, from pink (indicating traces of oxygen) to colourless (indicating absence of oxygen).

2. Isolation of Anaerobic Bacteria from Spent MWFs

(1) Sample Preparation for Initial Isolation

The purpose of the sample preparation was to increase the biomass of the anaerobic bacteria colonising the samples of spent MWFs for initial isolation. The spent MWFs were diluted to 1% or 2%v/v, adjusting the pH to 7. Standard serum bottles (Sigma-Aldrich, 33110) with a total volume of 100ml and a working volume of 80ml were used for sample preparation. Each serum bottle contained a 16ml diluted spent MWF sample with 1ml trace element solution (see Appendix 6 for details) and 63ml mineral buffer medium (see Appendix 6). The serum bottles were flushed with N_2 gas for three minutes, sealed with rubber seals (Fisherbrand, 12991231), and then incubated at 35℃ for one month.

(2) Bacteria Isolation and Purification

After one month's incubation, the samples were taken from the serum bottles and poured onto the surface of the COD-reducing bacteria and methanogen selective agar media (see Appendix 6) respectively. The media were kept in petri dishes (Fisherbrand, FB51504). The prepared plates were kept in anaerobic jars (Sigma-Aldrich, 28029) with a gas kit (AnaeroGen, Oxoid) at 35℃ for two weeks. Then, all the single colonies of anaerobic bacteria that had grown on the petri dishes were picked off for further purification by employing the following method (Sharma, 2007, p. 168):

- Prepare bacterial suspensions of the colonies by mixing the sample in 5ml phosphate buffered saline (PBS, Sigma-Aldrich, P5493).

- Take 1ml of each bacterial suspension and inoculate it on a petri dish containing the selective agar media.
- Incubate the petri dishes in the anaerobic jar with a gas kit at 35℃ for one week, and then observe the colonies. Discard plates containing overlapping or diffused colonies.
- Select plates showing different types of distinct, individual well-separated colonies.
- Pick up the selected colonies and mix in 5ml PBS, repeat the procedure from the beginning till all colonies are of same colour, type, etc.

The pure strains isolated from the agar plates were mixed with equal volumes of 80% glycerol (Sigma-Aldrich, G5516) in Eppendorf tubes (Sigma-Aldrich, Z768744) and kept in a -20℃ freezer for further experiments.

5.2.2 Results and Discussion

In total, 65 pure strains of COD-reducing bacteria were isolated from the samples of spent MWFs provided by Microbial Solution Ltd; six from the spent RELUBRO 800 sample, three from the Hysol 113 Coll sample, eight from the AETC 3380 sample, two from the Rolls Royce 7/6/10 sample, 10 from the F22 system sample, five from the DK014S-24, 11 from the T3 Cell3 Oil sample, four from the Bridgeport 10-355, eight from the Hongh Tong Mix Tank sample, six from the STA-PUT G68 sample, and two from the Hysoh Mix sample. Figure 5.3 shows several examples of the unidentified pure COD-reducing bacteria strains isolated from the spent MWFs.

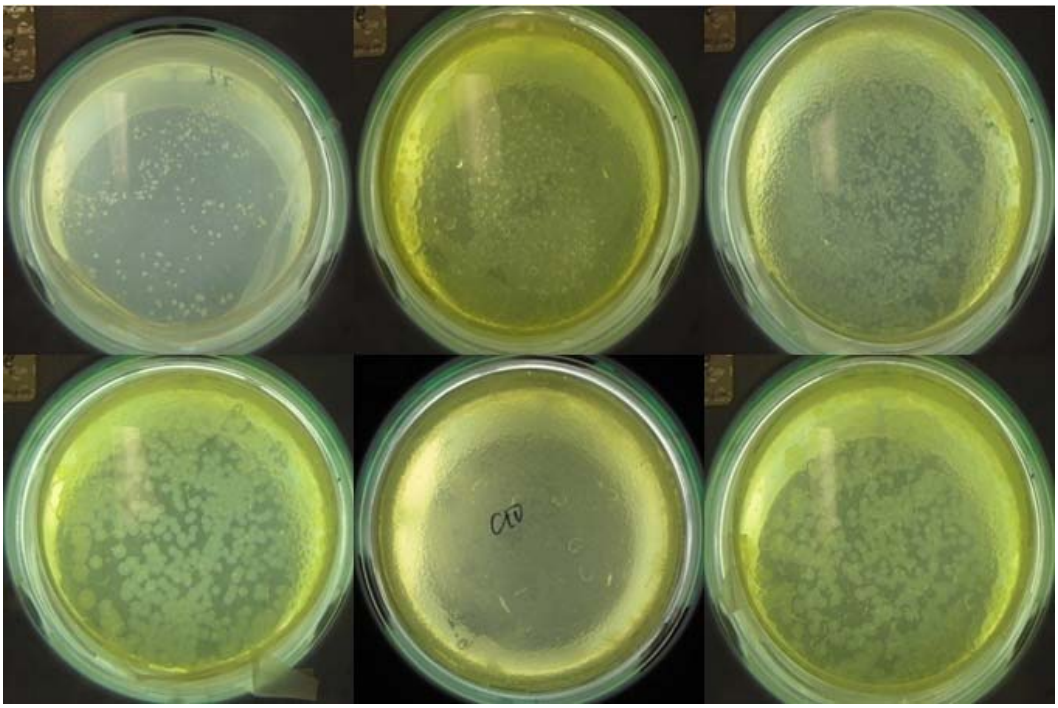

Figure 5.3: Six examples of the COD-reducing bacteria isolated from the samples of the spent MWFs using anaerobic selective agar medium

In section 5.4 below, 29 of the 65 strains, which had relatively higher COD reduction rates in RELUBRO MWF, were selected for genetic sequencing (to be described in

section 5.3) and subsequent studies. In all, 19 different species were identified. To sum up, combining the final outcome of the genetic sequencing (to be detailed in section 5.4.2), the result of the bacteria-isolation work in this study demonstrates the high level of biodiversity in the anaerobic bacteria colonising in spent MWFs. Thus, these isolated COD reducers may be exploitable in terms of overcoming the toxicity of the RELUBRO MWF in the anaerobic degradation process. In contrast, it was also noted that the experiment failed to isolate any anaerobic bacteria from eight of the 20 spent MWF samples. These eight samples contained high-concentrated petroleum compounds, which may have been too toxic to colonise any anaerobic bacteria, or their biodegradability have simply been too low to support the growth of anaerobic bacteria.

In this study, there were two cultures containing methanogens isolated from the spent MWFs using methanogen selective agar medium (see Appendix 6). The two cultures were named M1 (from the T3 Cell3 Oil sample) and M2 (from the Hongh Tong Mix Tank sample) in subsequent studies (shown in Figure 5.4, A & B).

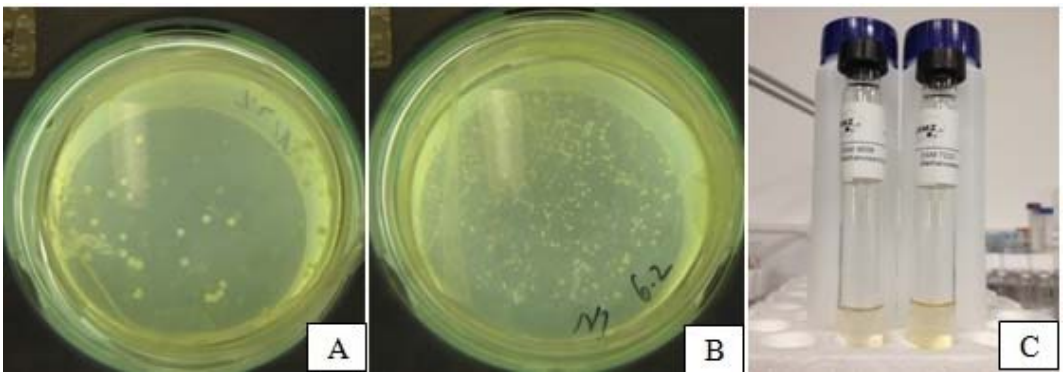

Figure 5.4: Two mixed colonies, named M1 and M2 (A & B), isolated from spent MWFs which contain methanogens, and the two pure methanogen strains purchased from DSMZ (C)

The existence of methanogens in the two cultures was confirmed using the 16s rRNA FISH probe (see section 4.2.2). However, the attempt to purify the methanogen strains in the cultures failed using the bacteria-isolation approach described above in this section.

According to previous studies (Hungate, 1950; Xun et al. 1990; Ariesyady et al., 2007; Hoj et al., 2008), methanogens are obligate anaerobic bacteria; a minute amount of oxygen can kill them. The experimental condition, only including an anaerobic jar and bag, was not able to guarantee strict anaerobic operating conditions for the whole bacteria-isolation process.

Two pure strains of *Methanosaricina mazei* were purchased (DSMZ, Germany) as standard for subsequent studies. The product codes of the two strains are DSM 7222 and DSM 4556 (see Figure 5.4, C above). The *Methanosarcina mazei* strains were selected for this study because they are one of the major methanogen species and are commonly found in previous studies regarding the anaerobic degradation process (Yu et al., 1997; Yamaguchi and Minami, 1998; Medalia et al., 2006; Rastogi et al., 2008; Vrieze et al., 2012; Godin et al., 2012; Kim et al., 2012). According to the culture information provided by the supplier, DSM 7222 was isolated from digested sewage sludge and DSM 4556 from contaminated mud in an oil drilling site, which had a similar environment containing petroleum compounds such as MWFs. In this part of the study, the purchased strains represented standard methanogen strains and the two mixed cultures represented the methanogen culture we detected in spent MWFs.

5.3 Selection of the Isolated COD-reducing Bacteria

The isolation of anaerobic bacteria from spent MWFs resulted in 65 COD reducer strains being obtained in total. These strains were isolated from different MWFs and not all of them were likely to adapt to the toxic conditions of RELUBRO MWF. Therefore, a selection procedure was carried out aiming to select the isolated COD-reducing bacteria that had relatively greater activity in terms of a COD reduction rate in RELUBRO MWF and to reduce the number of strains for subsequent studies.

5.3.1 Methods and Materials

All the COD-reducing bacteria strains isolated from the spent MWFs were cultivated in the enrichment broth of COD reducers (see Appendix 6) for three days and diluted to sample optimal density (1Abs) at 600nm (OD_{600}) using a PBS buffer. Ten ml of each sample was centrifuged at 12,000rpm for five minutes, and the top liquid layer was discarded. The bacteria pellet was re-suspended in a 10ml diluted MWF sample (2,000mgCOD/L, pH 7) previously sterilised using a 0.2µm syringe filter with 1mg/L resazurin added. Then, the samples were transferred into culture vials (12ml total volume) that were flushed with N_2 gas for three minutes. The prepared vials were kept at 35℃ for five days. The COD values of the raw mixed samples and after a period of incubation were measured in order to determine the biodegradation rates over five days. All samples were carried out in triplicate.

5.3.2 Results and Discussion

The COD reduction rates of the 65 isolated COD reducers in the RELUBRO MWF sample (2,000mg/L, pH 7) over five days are shown in Table 5.1 below. No methane was detected in any of the samples, which suggests that there was no methanogen in the samples.

Table 5.1: Mean COD reduction rate of the MWF sample (2,000mgCOD/L, pH 7) over five days inoculated with the individual isolated COD-reducing bacteria (n=3).

Strain Code	Mean COD Reduction (%)	Strain Code	Mean COD Reduction (%)	Strain Code	Mean COD Reduction (%)	Strain Code	Mean COD Reduction (%)
C1	3.1±0.5	**C18**	12.4±0.1	**C35**	8.3±0.4	**C52**	8.6±0.5
C2	3.6±0.1	**C19**	11.2±0.5	**C36**	2.3±0.3	**C53**	11.3±0.3
C3	4.2±0.5	**C20**	10.3±0.2	**C37**	14.2±0.2	**C54**	17.2±0.5
C4	3.1±0.1	**C21**	11.4±0.1	**C38**	26.2±0.5	**C55**	22.6±1
C5	5.5±0.2	**C22**	12.1±0.1	**C39**	5.7±0.6	**C56**	29.1±2.5
C6	9.2±0.4	**C23**	6.1±0.2	**C40**	6.7±0.3	**C57**	14.5±0.4
C7	23.1±0.1	**C24**	0.9±0.1	**C41**	11.2±0.3	**C58**	16.4±0.8
C8	8.7±0.2	**C25**	4.9±0.5	**C42**	1.2±0.1	**C59**	12.1±1.1
C9	5.1±0.5	**C26**	0.1±0.2	**C43**	20.2±0.8	**C60**	10.2±0.7
C10	16.8±0.2	**C27**	2.8±0.2	**C44**	0.8±0.1	**C61**	0.8±0.3
C11	1.1±0.1	**C28**	10.7±0.5	**C45**	38.1±1	**C62**	10.1±1
C12	7.5±0.1	**C29**	2.2±0.3	**C46**	12.8±0.3	**C63**	6.1±0.4
C13	10.1±0.3	**C30**	4.7±0.2	**C47**	23.7±0.7	**C64**	0.7±0.4
C14	5.4±0.2	**C31**	2.1±0.2	**C48**	6.9±0.3	**C65**	10.9±0.4
C15	11.4±0.2	**C32**	3.1±0.1	**C49**	6.3±0.3		
C16	9.9±0.2	**C33**	2.8±0.2	**C50**	22.3±0.9		
C17	6.9±0.1	**C34**	2.1±0.2	**C51**	10.3±0.8		

As shown above in Table 5.1, the COD reduction rates of 29 strains were above 10% in this five-day experiment, including 11 strains with 15% COD reduction rates or higher. The results suggest that some of the COD-reducing bacteria isolated from other spent MWFs had adapted to the toxicity of RELUBRO MWF and maintained a relatively greater activity in the toxic environment, a finding consistent with previous studies of the microbiology of spent MWFs (Baker et al., 1983; Robinson, 1986; Asakawa et al., 1995; Cheng et al., 2005). This suggests the COD reducers were less sensitive to the toxicity of RELUBRO MWF, which could explain why so few methanogens were isolated from spent MWFs, since they are too sensitive to toxicity.

The 29 strains with the top COD reduction rates were selected and analysed by exploring their genetic sequencing using the molecular biology methods described in section 5.4 below.

5.4 Genetic Sequencing Analysis of the Isolated Anaerobic Bacteria

The anaerobic bacteria isolated from spent MWFs were identified in this part of the study by genetic finger printing (Lane, 1991). This was achieved by employing four basic steps: genomic DNA extraction, agarose gel electrophoresis, polymerase chain reaction (PCR) amplification, and DNA sequencing.

5.4.1 Methods and Materials

1. Genomic DNA Extraction

The protocol of the genomic DNA extraction employed in this study is given in Appendix 4. Optimisation of the splitting time with sodium dodecyl sulphate (SDS) in the protocol

was carried out using a standard methanogen strain-DSM 7222 *Methanosarcina mazei*. The genomic DNA samples of DSM 7222 *Methanosarcina mazei* extracted with four different splitting times were run in agarose gel, for 0.5 hours, one hour, two hours and three hours. The result of the agarose gel electrophoresis is shown in Figure 5.3.

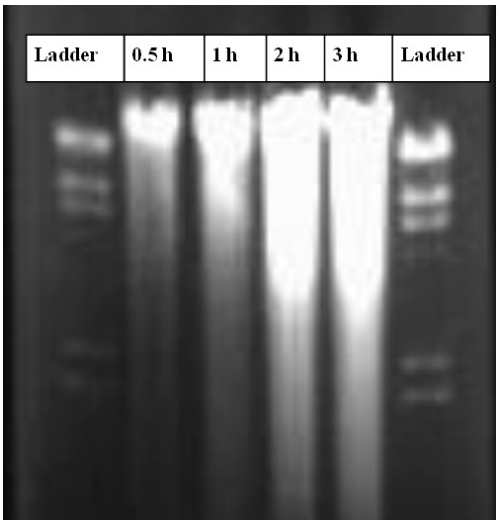

Figure 5.5: The extracted genomic DNA samples of DSM 7222 splitting with SDS for 0.5h, 1h, 2h and 3h

2. Agarose Gel Electrophoresis

The agarose gel electrophoresis works in this study were carried out using a Bio-Rad Mini Sub-Cell system with a power supply (Bio-Rad Power PAC Basic). The reagents used for agarose gel electrophoresis include: Gel loading buffer (G2526, Sigma-Aldrich); Agarose gel (UltraPureTM, Life Technologies); SYBR gold nucleic acid gel stain (10,000X Concentrate in DMSO, Invitrogen, Life Technologies); DNA ladder (BIOLINE HyperLadder I, Fisher Scientific); and TAE buffer (50X, UltraPureTM, Life Technologies). The voltage of the gel electrophoresis was 90V.

3. PCR Amplification

The PCR amplification procedure is given in Appendix 5. The PCR primers used in this study are given in Table 5.2 below.

Table 5.2: Primers used for PCR amplification works

Primers	Primer Sequence	Reference
MLf	GGTGGTGTMGGATTCACACARTAYGCWACAGC	Ferry, 1993
MLr	TTCATTGCRTAGTTWGGRTAGTT	Ferry, 1993
63F	CAGGCCTAACACATGCAAGTC	Osborn et al., 2000
519R	GTATTACCGCGGCTGCTG	Hutter et al., 2002
8F	AGAGTTTGATCCTGGCTCAG	Lane, 1991
1492R	GGTTACCTTGTTACGACTT	Lane, 1991
25F	AGAGTTTGATCMTGGCTCAG	Dojka et al. 1998
1391R	GACGGGCGGTGTGTRCA	Barns et al., 1994

The PCR amplification products were extracted using a gel extraction kit (GenElute, Sigma-Aldrich) from the agarose gels for the DNA sequencing work.

Following the PCR procedure described in *Molecular Cloning: A Laboratory Manual (Fourth Edition)* (Green and Sambrook, 2012, pp. 455–540), optimisation of the amount of DNA template and primer concentration was carried out with a sample of DSM 7222 *Methanosarcina mazei*. As displayed in Figure 5.4 below, the PCR products amplified with 5ng and 10ng of the DNA template were stained in an agarose gel. It can be seen from Figure 5.4 that the band using the 10ng DNA template had a stronger luminance

signal. Thus, 10ng of DNA template to be used in each PCR reaction was selected for this study.

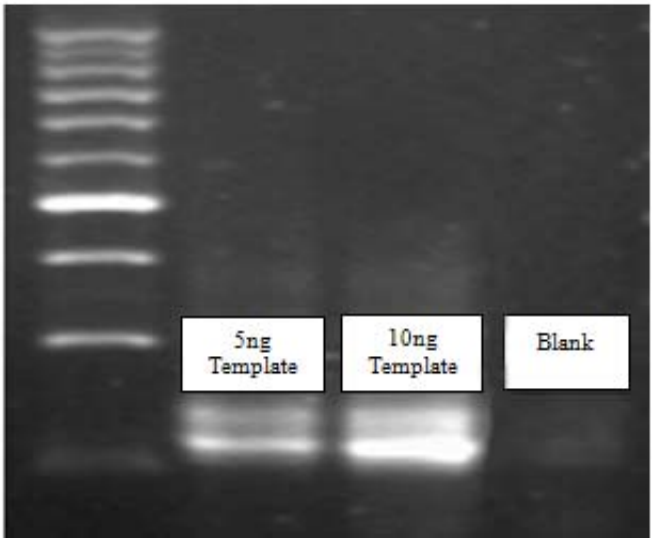

Figure 5.6: The stained PCR products amplified with 1µl and 5µl DNA templates in an agarose gel

Figure 5.7 below shows the stained PCR products amplified with 5ng, 25ng and 50ng primers in each reaction. It demonstrates that the band amplified with 50ng primers has a stronger luminance signal, which entailed that 50ng primers were used for the PCR reactions in this study.

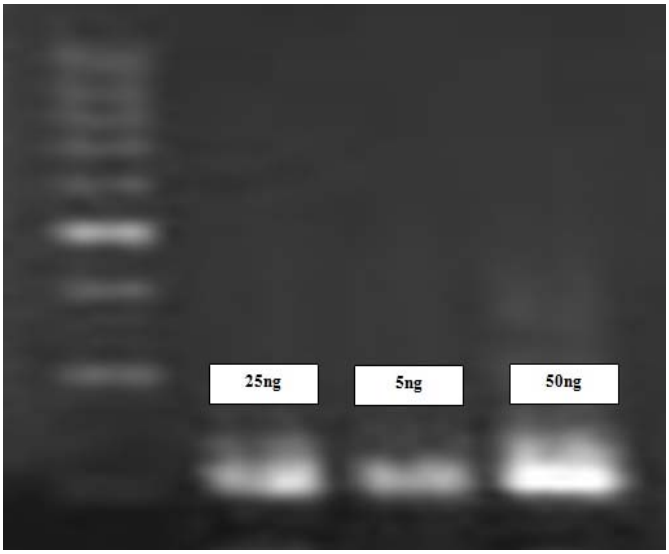

Figure 5.7: The stained PCR products amplified with 5ng, 25ng and 50ng PCR primers in an agarose gel

4. DNA Sequencing and Bacteria Identification

The DNA sequencing service used in this study is provided by Sourcebioscience (see www.lifesciences.sourcebioscience.com). The results of the DNA sequence have been checked against an online database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the isolated bacteria.

5.4.2 Results and Discussion

As a result of the genetic works described in section 5.4, 29 strains of the COD reducer isolates with best recorded COD reduction rates for RELUBRO MWF (over 10% COD reduction over five days). Thus, these 29 strains were identified by analysing their 16s rRNA sequences. The results are given in Table 5.3 (See Appendix 9 for the details of sequences of the strains) :

Table 5.3: List of identified COD-reducing bacteria isolated from spent MWFs

No.	Strain Code	Name of the Species
1	C45	*Anaerobacillus*
2	C10	*Enterobacter aerogenes*
3	C28,C59 & C62	*Bacillus sp.*
4	C53	*Bacillus firmus*
5	C38,C43 & C50	*Clostridium sporogenes*
6	C55	*Clostridium perfringens*
7	C54	*Clostridium pasteurianum*
8	C7&C60	*Clostridium sulfidigenes*
9	C21	*Citrobacter freundii*
10	C46	*Desulfovibrio baculatus.*
11	C22	*Enterococcus.sp.*
12	C18,C51,C57 &C65	*Escherichia coli*
13	C56	*Micrococcus flavus*
14	C20	*Pasteurella sp.*
15	C15	*Proteus vulgaris*
16	C19, C47 & C58	*Pseudomonas sp.*
17	C13 & C41	*Rhodocyclus sp.*
18	C37	*Trabulsiella guamensis*

The phylogenetic tree, as displayed in Figure 5.8 below, shows the relationships among the identified COD-reducing bacteria. It can be seen from the phylogenetic tree chart that the COD-reducing bacteria species identified by genetic fingerprinting include obligate

(such as *Desulfovibrio sp.*, *Anaerobacillus sp.* and *Clostridium sp.*) and facultative anaerobes (such as *Enterococcus sp.*, *Pseudomonas sp.* and *Escherichia sp.*). Some of the obligate anaerobes were also found in previous studies (van der Gast et al., 2001; 2003a; 2003b; 2004) of the microbiology of wasted and in-use MWFs, including *Pseudomonas sp.*, *Citrobacter sp.* and *Enterococcus sp.*

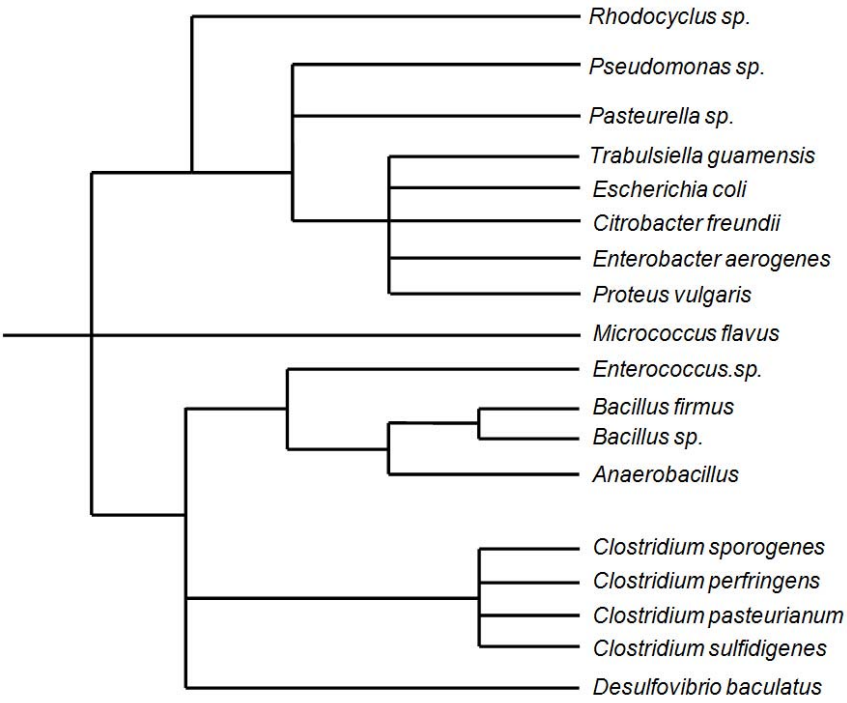

Figure 5.8: Phylogenetic tree of bacteria sequences retrieved from the COD-reducing bacteria as determined by the neighbour-joining method on the basis of the 16s rRNA sequence

The results of bacterial identification confirmed the high level of biodiversity in the anaerobic bacteria colonising in spent MWFs. This degree of diversity was suggested that there was a real possibility of assembling an anaerobic bacteria community which is capable of overcoming the MWF toxicity and treating waste MWFs.

In order to simplify the analysis of the subsequent studies, the number of the COD reducer strains exploited in the subsequent studies was narrowed down. Only one strain, which had the highest COD reduction rates (shown in Table 5.1) in RELUBRO MWF, was selected from each identified species shown in Table 5.3. Compared with other species, the *Clostridium sporogenes* strains had relatively greater COD reduction rates; thus, two of them with the greatest COD reduction rates were selected for subsequent studies. Thus, based on the rationale, the number of the COD reducer strains exploited in the subsequent studies was narrowed down from 29 to 20 and they were exploited for the subsequent studies regarding overcoming the toxicity of RELUBRO MWF. These were: C7 (*Clostridium sulfidigenes*), C10 (*Enterobacter aerogenes*), C13 (*Rhodocyclus sp.*), C15 (*Proteus vulgaris*), C18 (*Escherichia coli*), C19 (*Pseudomonas perolens*), C20 (*Pasteurella sp.*), C21 (*Citrobacter freundii*), C22 (*Enterococcus.sp.*), C28 (*Bacillus sp.*), C37 (*Trabulsiella guamensis*), C38 (*Clostridium sporogenes*), C43(*Clostridium sporogenes*), C45 (*Anaerobacillus*), C46 (*Desulfovibrio baculatus*), C47 (*Pseudomonas sp.*), C53 *(Bacillus firmus*), C54 (*Clostridium pasteurianum*), C55 (*Clostridium perfringens*), and C56 (*Micrococcus flavus*).

5.5 Optimising the Experimental Conditions for Exploiting the Isolates

In the key outcome of the analysis detailed in section 5.5 above, was that 20 identified COD-reducing bacteria strains isolated from spent MWFs were selected to be exploited in the treatment of RELUBRO MWF for overcoming its toxicity and making it more conducive for methanogens to generate more methane. It was important to determine the optimal incubation conditions of the selected COD reducer and methanogen in order to maximise their function for the anaerobic degradation of RELUBRO MWF, i.e. COD reduction and methane generation. In particular, it was important that the COD reduction resulted in the toxicity of the MWF being reduced, which was established in the experiment regarding employing a biosensor to measure the toxicity of RELUBRO MWF (as outlined in Chapter 4). Therefore, in this part of the study, it was important to determine the optimal incubation conditions, including the pH value and temperature for growth and maximising activity of the isolated COD-reducing bacteria and methanogen strains.

pH is an important factor that needs to be considered when optimising the performance of a microbial system. Cheng et al. (2005) point out that the high pH of MWFs maintained by alkaline compound formulations enhanced biocide activity. van der Gast et al. (2004) investigated the effects of pH on the aerobic treatment process of MWFs and their results suggested that the optimal pH range for the biological treatment of MWFs is between 6 and 7.

As would be expected the incubation temperature affects the activity, diversity and community structure of anaerobic microorganisms (Boone, 1987; Lone et al., 2008),

especially that methanogens which are very sensitive to changing pH and temperature (Anderson and Yang, 1992; van Haandel and Lettinga, 1994; Xie and Liu, 2007). In general, the bacterial activity of anaerobic bacteria was relatively greater in the range of 20℃～50℃ (El-Mashad et al., 2004). Deepak et al.′s study (1994) demonstrated in an increase in COD reduction rate as the temperature increased from 15℃ to 30℃ in a study regarding the biodegradation kinetics of MWF, and Cheng et al. (2004) found that the rate at 40℃ was twice that as recorded at 30℃.

Four species of identified COD-reducing bacteria strains (i.e. C38 (*Clostridium sporogenes*), C47 (*Pseudomonas sp.*), C55 (*Clostridium perfringens*) and C56 (*Micrococcus flavus*)) were selected in this experiment because they had the greatest COD reduction rates in the RELUBRO MWF sample (2,000 mgCOD/L pH 7) over five days incubation (described in section 5.4). The bacteria-isolation procedure presented in section 5.3 resulted in two mixed cultures isolated from spent MWFs, which were named M1 and M2. These two cultures could generate methane from media; however, the attempt to purify the methanogen strains in the cultures failed. Therefore, two standard methanogen strains were purchased from DSMZ: *Methanosarcina mazei* DSM 7222 and DSM 4556, so that at least the potential of pure strain to produce methane from the MWF′s degradation process could be investigated. The *Methanosarcina mazei* strains were selected for this study because they are one of the major methanogen species reported to be popular in studies regarding microbiology in anaerobic wastewater treatment processes (Yu et al., 1997; Yamaguchi and Minami, 1998; Medalia et al., 2006; Rastogi et al., 2008; Vrieze et al., 2012; Godin et al., 2012; Kim et al., 2012). In this part

of the study, the purchased strains represent standard methanogen strains and the two mixed cultures represent the methanogen cultures that were indigenous to MWFs.

5.5.1 Methods and Materials

1. Effect of Initial pH on Activity of the COD-reducing Bacteria

pH values in the range between 5 and 9 were selected, which is the typical range employed for biological wastewater treatment processes (van Haandel & Lettinga, 1994; Anderson & Yang, 1992; Visser et al., 1993; van der Gast et al., 2004). Thus, the seven pH values were selected in this experiment, including 5, 6, 6.5, 7, 7.5, 8 and 9. In addition, the cultures of the selected COD reducer strains were mixed MWF samples at the different pH values, although it should be noted that there was no way to ensure the final pH value of the mixed sample could be exactly as set at these seven values. Thus, the final pH values of the mixed samples were measured and an error range of ±0.2 for each pH value was allowed around the selected pH values. The COD reducer strains were incubated in the enrichment broth medium for five days, and the cells were collected using a centrifuge at 120,000rpm for five minutes. The bacteria pellets were then diluted to the same OD_{600} (1Abs) using a PBS buffer. Forty ml of each diluted sample was transferred into three 15ml centrifuge tubes (Sigma-Aldrich, T1943) and centrifuged at 120,000rpm for five minutes. The PBS buffer in the tubes was discarded; the bacteria pellets were re-suspended in 40ml diluted MWF samples (2,000mgCOD/L) at seven different pH values. The final pH values of the seven samples were as follows: 5.08, 5.94, 6.59, 7.10, 7.58, 8.08 and 9.12. The samples were kept in 50ml centrifuge tubes which were sealed after flushing with N_2 for five minutes. These were kept in a shaking

incubator at 35℃ for three days. The COD reduction of each sample over the three-day experiment was measured for analysis. Blank MWF samples with different pH values were prepared as controls.

2. Effect of Temperature on the Activity of the COD-reducing Bacteria

In terms of sample preparation, the same procedure described in the section above (5.5.1.1) was employed in this experiment. The bacteria pellets were re-suspended in diluted MWF sample (2,000mgCOD/L, pH 7). The samples were incubated at 20℃, 30℃, 35℃, 40℃ and 50℃ for three days, and the COD reduction rate of each sample measured. Blank MWF samples incubated at the different temperatures were used as controls.

3. Effect of the Initial pH on the Activity of Methanogens

The pH values were selected in the range between 5 to 9, which has been widely investigated for biological wastewater treatment processes in previous studies (van Haandel & Lettinga, 1994; Anderson & Yang, 1992; Visser et al., 1993; van der Gast et al., 2004). Thus, the nine pH values were selected in this experiment, including 5, 5.5, 6, 6.5, 7, 7.3, 7.8, 8.2 and 9; and an error range of ±0.2 at each pH value was allowed in the mixed samples containing methanogen cultures and growth medium. Ten ml of the methanogen growth medium (see Appendix 6) was added to a glass vial (12ml, Sigma-Aldrich, Z251070) with 0.1g methanogen inocula prepared following the procedure given in Appendix 7. Nine methanogen media samples with the same concentration and different pH levels were prepared. The final pH values of the medium samples were as

follows: 5.06, 5.42, 6.02, 6.58, 7.04, 7.31, 7.84, 8.23 and 9.04. The vials were flushed with N_2 gas for three minutes and sealed with rubber seals. Then, the sample vials were incubated at 35℃ for one week and the concentration of methane generated in each vial was measured.

The Shimadzu GC 2010 Plus system was used to carry out the qualitative and quantitative analysis of methane in the biogas samples with a HP-PLOT/U column (Agilent Technologies) and a FID detector.

4. Effect of Temperature on the Activity of Methanogens

The samples were prepared using the same method described in the section above (5.6.1.3). Five temperatures were chosen for the experiment: 20 ℃, 30 ℃, 35 ℃, 40 ℃ and 50℃. The sample vials were incubated for one week and the concentration of methane generated in each vial was measured using the Shimadzu GC system introduced above.

5.5.2 Results and Discussion

1. Effect of Initial pH on the Activity of the COD-reducing Bacteria

Figure 5.9 below represents the changes in the mean COD reduction rates in the MWF samples (2,000mgCOD/L) individually inoculated with the four COD reducer strains over three days at different initial pH of the MWF.

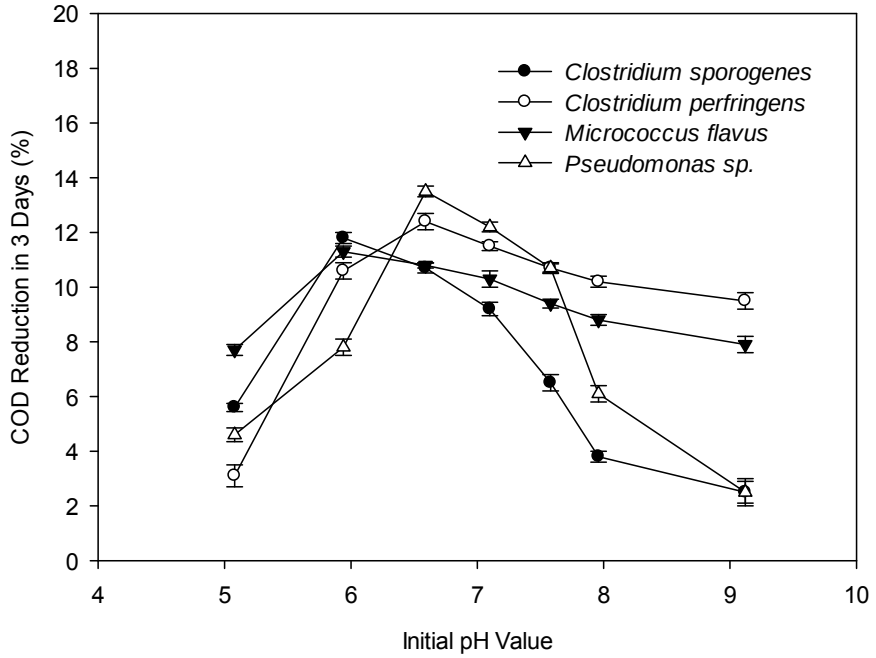

Figure 5.9: Changes in the mean COD reduction efficiencies of four isolated COD reducers at different initial pH of the MWF (2,000mgCOD/L, three days, n=3, Error Bar: SD, 35℃)

As can be seen from Figure 5.9, in general the COD-reducing bacteria had greater activity in the pH range between 6 and 7. It should also be noted that some of the COD reducers, such as *Clostridium perfringens* and *Micrococcus flavus*, still had relatively good activity in the higher pH range; this could explain why they were able to survive in

spent MWFs which had a pH range of 8 to 11 in general (Perez et al., 2006; Anderson et al., 2003).

2. Effect of Temperature on the Activity of the COD-reducing Bacteria

Figure 5.10 below shows the results of the changes in COD reduction rates in the RELUBRO MWF sample (2,000mg/L, pH 7) inoculated individually with the four COD isolates that had the greatest activity in RELUBRO MWF in the selection experiment (see section 5.4 for details). The four isolates were: C38 (*Clostridium sporogenes*), C47 (*Pseudomonas sp.*), C55 (*Clostridium perfringens*) and C56 (*Micrococcus flavus*).

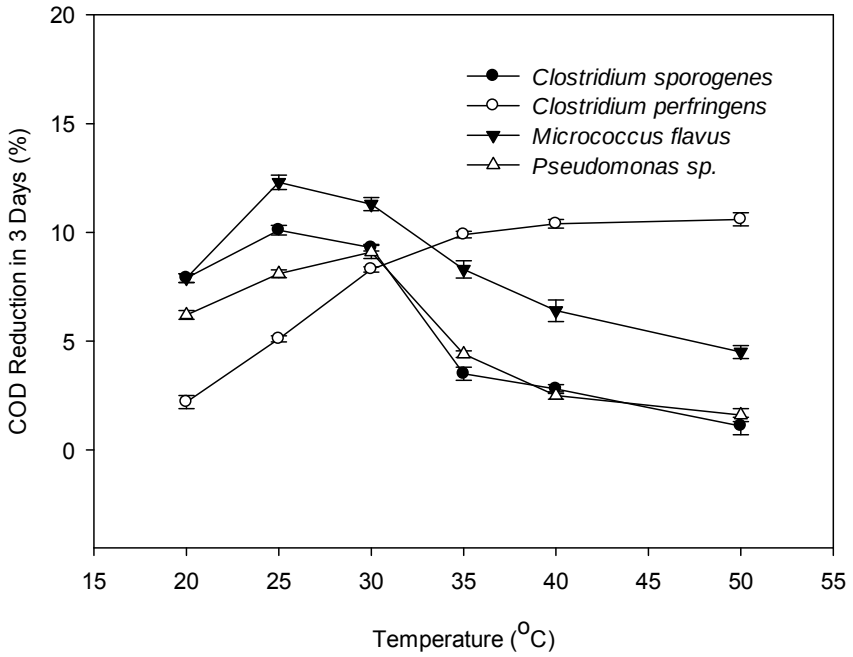

Figure 5.10: Changes in the mean COD reduction efficiencies of four isolated COD reducers at different incubation temperatures (MWF: 2,000mgCOD/L, three days, n=3, Error Bar: SD)

Figure 5.10 shows that three of the four bacteria had relatively greater activity in terms of COD reduction rates at around room temperature (20℃~30℃). However, the strain

Clostridium perfringens had relatively greater activity in the higher temperature range which plateaued at 35℃.

3. Effect of the Initial pH on Activity of Methanogens

The two standard methanogen strains (DSM 7222 and DSM 4556) and the two mixed cultures (M1 and M2) isolated from spent MWFs were incubated in the methanogen broth medium at different initial pH values for two weeks. The volumes of the methane produced from the samples are shown in Figure 5.11 below.

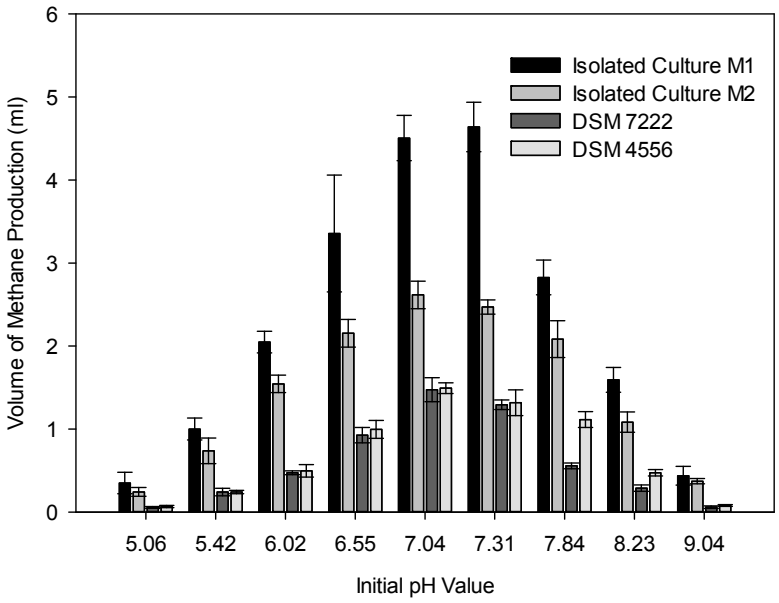

Figure 5. 11: Mean volume of methane production of the two methanogen cultures isolated from waste MWFs (M1 & M2) and two purchased methanogen strains (DSM 7222 & DSM 4556) from the growth medium at different initial pH values (n=3, Error Bar: SD, 35℃)

The Figure shows that, in general, the methanogens had a greater methane production rate in the initial pH range between 6.5 and 7.3, which suggests the same optimal pH range for the growth of methanogens reported in previous studies (Anderson and Yang,

1992; van Haandel and Lettinga, 1994). It can also be seen that the strains, DSM 7222 and DSM 4556, had similar capabilities in terms of methane production rate, but that the DSM 4556 strain had greater activity at around pH 8. This may suggest that the DSM 4556 strain, which was isolated from the mud in an oil drilling site, was better adapted to habitats and substrates enriched in petroleum chemicals.

4. Effect of Temperature on the Activity of Methanogens

Figure 5.12 below shows the results of methane production rates of the four methanogen strains over seven days incubation at five different temperatures.

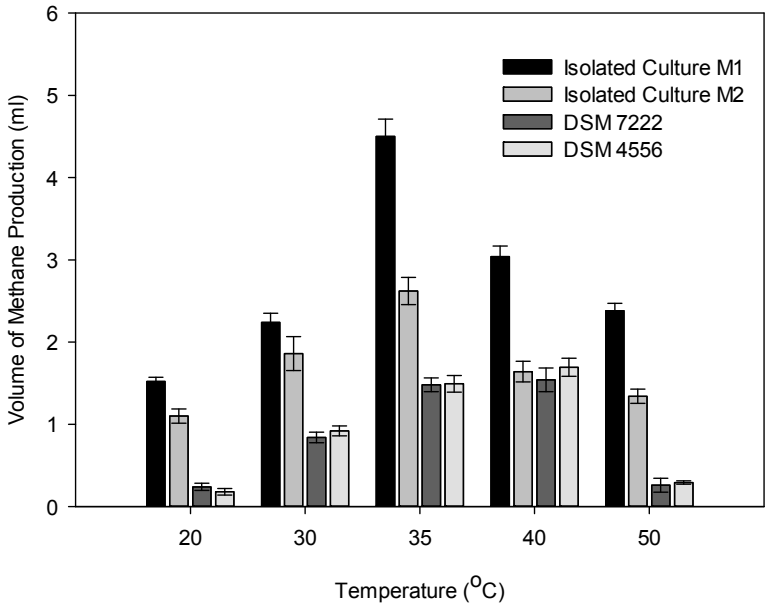

Figure 5.12: Mean volume of methane production of the two methanogen cultures isolated from waste MWFs (M1 & M2) and two purchased methanogen strains (DSM 7222 & DSM 4556) from the growth medium at different temperatures (n=3, Error Bar: SD, t=7d)

It can be seen from Figure 5.12 that all of the methanogen cultures, i.e. both the two standard strains (DSM 7222 & DSM 4556) and the two mixed cultures isolated from

waste MWFs, had greater methane production in the temperature range between 30℃ and 40℃; their methane production rates at 20℃ and 50℃ were much lower than that at other temperature scales, especially the two pure strains of methanogen. Specifically, the two mixed cultures containing methanogens (M1 & M2) had the greatest methane production rates at 35℃. The two standard methanogen strains had relatively greater methane production rates at both 35℃ and 40℃, while there were no significant differences between the results at the two temperatures (the p-values of the data's t-Tests at 35℃ and 40℃ were 0.87 and 0.41 respectively, which can be taken to be a statistically significant difference when the p-value of the t-Test was set at 0.05 or below in this study). This may suggest that, in this study, the optimal temperature for the incubation of the methanogens strains in subsequent studies was around 35℃, as suggested by Xie and Liu (2007) and Cheng et al.'s review (2005).

5. Summary and Discussion.

To sum up, the results shown in Figure 5.9 and 5.11 suggest that the optimal pH ranges for incubating the isolated COD reducers and methanogens were in the range of 6～7 and 6.5～7.3, respectively. Thus, a pH scale that was both appropriate for exploiting the COD isolates and methanogens was selected at 7, and this finding is in agreement with previous findings regarding biological treatment of MWFs (van der Gast et al., 2004, Cheng et al., 2005), which showed that the optimal pH range for the biological treatment of MWFs is in the range between 6 and 7.

Temperature plays an important role in anaerobic degradation as in all biological processes, impacting on the activity, biodiversity and community structure of anaerobic microorganisms, especially of methanogens (Boone, 1987; Kim et al., 2004; Lone et al., 2008). The findings shown in Figure 5.10 demonstrate that most of the COD reducers had relatively greater activity in the temperature range of 20℃ to 30℃, and the methanogens used in this study required an optimal temperature range of 30℃ to 40℃ for incubation. However, there was no conflict in choosing 35℃ as the standard temperature in this study. This is because it should be noted that the COD reducers with the greatest activity at room temperature range also demonstrated greatest activity at 35℃. Furthermore, COD-reducing bacteria and methanogens have different growth rates, but they are able to naturally reach a balance in the natural environment (Appels et al., 2008). In this study, it was challenging to assemble a mixed culture that was able to maintain a balance for the degradation of MWFs using the isolated bacterial strains. Thus, COD reducers and methanogens were employed separately to overcome the toxicity of the MWF and the methane produced.

In addition, it can be seen from Figure 5.11 and Figure 5.12 that the two cultures isolated from waste MWFs in this study had much greater methane production rates than the two standard pure strains purchased from the DSMZ culture collection. This suggests that the cultures isolated from waste MWFs may contain some other anaerobic bacteria that were helpful in terms of methane generation in the anaerobic degradation process. In this study, we had no success in attempting to separate the bacteria contained in the two cultures (M1 and M2) isolated from waste MWFs, which suggests that these bacteria

were very closely inter-dependent. Thus, no certain conclusion regarding their compositions and the interactions between methanogens and other bacteria could be made in this vein. This was also the major reason that two standard methanogen strains (DSM 7222 & DSM 4556) were purchased from a commercial culture collection to be used in the subsequent studies. Because of the complexity and technical limitations of separating different bacterial species in an anaerobic ecosystem, no certain conclusion has been drawn, just like was the case in previous studies regarding the classification of anaerobic bacteria (Bryant, 1979; Appels et al., 2008).

5.6 Overcoming the Toxicity of the RELUBRO MWF Using the Isolates

In this part of the study, the characterised COD reducers isolated from spent MWFs were used to assemble a mixed consortium for exploitation, specifically for reducing the toxicity of RELUBRO MWF. The rationale was that if the selected COD reducers were efficient in terms of degrading the toxic/low-biodegradable compounds in RELUBRO MWF, this would help to overcome toxicity, which could subsequently result in enhancement of methane generation from the MWF.

There have been some studies aimed at degrading spent MWFs using chemical treatment methods, such as an advanced oxidation process, and biological methods (Rios et al., 1998; Yoshio & Masanori, 2001; van der Gast et al., 2001; Yuan et al., 2011; Jagadevan et al., 2011; 2012). Most of the previous studies regarding the biological treatment of MWFs focused on aerobic treatment and few cases attempted anaerobic treatment processes because of the toxicity of MWFs (Cheng et al., 2004; 2005). Thus, there are no reported studies that focus on overcoming the toxicity of MWFs by inoculating specific

anaerobic cultures and thus enhancing the treatment efficiency in terms of COD reduction and methane production rates. In contrast, studies assessing the biological treatment processes have focused on two aspects separately: the biodiversity of the anaerobic ecosystem and its influence on functionality in terms of the degradation of the organic components of MWFs (Hill, 1983; Jarvholm, 1981; Zeman et al., 1995; Moore et al., 2000; van der Gast et al., 2001; Khan and Yadav, 2004; Rhodes et al., 2008). However, the relationship between the biodiversity and functionality, although it is the key to anaerobic disposal approaches, has received very little attention (Loreau et al., 2001; Bell et al., 2005). This is mainly because of the complexity of anaerobic habitats, the fact that the various anaerobic bacteria could only be classified into categories according to their metabolic processes, and that the understanding in regard to the relationship and interactions between different species remains weak (Bryant, 1979; Appels et al., 2008).

The studies described in this section consist of two major parts: 1) the assembling of the mixed consortium; and 2) the treatment of RELUBRO MWF using the mixed COD reducing consortium to overcome its toxicity.

5.6.1 Methods and Materials

1. Assembling a Mixed Consortium Using the Selected Isolates

The freeze-dried stocks of the identified COD-reducing bacteria isolated from spent MWFs were re-suspended with distilled water and left at 25℃ for two hours. Then, the OD_{600} of cultures were diluted to 1Abs using the PBS buffer. Ten ml of each sample was used to mix with different samples for making mixed cultures of increasing complexity

from 1, 2, 3, 4, 5, 10, 15, and 20 species respectively. The samples for each combination were selected randomly. After diluting to the same OD_{600} value (1Abs), 20ml of the mixed cultures was centrifuged at 12,000rpm for five minutes. The bacteria pellets were re-suspended in the MWF sample (2,000mgCOD/L, pH 7) with equivalent volumes of the mixed cultures and 1mg/L resazurin. Then, 10ml of each of the prepared samples were transferred into culture vials (total volume: 12ml), and flushed with N_2 gas for three minutes until the resazurin was colourless. The prepared vials were kept on a shaking incubator at 35℃ at 80rpm for seven days. All the assessments were carried out in triplicate and a blank sample of MWF without added COD reducers was prepared. The final mixed culture of the COD-reducing bacteria was assembled for further experiments investigating ways of overcoming the MWF's toxicity using the mixed consortium.

2. Overcoming the Toxicity of RELUBRO MWF Using the Assembled Consortium

The freeze-dried stock of the mixed culture of the COD-reducing bacteria was re-suspended with distilled water and left at 25℃ for two hours. The OD_{600} of the culture was diluted to 1Abs using the PBS buffer. Then, 50ml of the mixed culture was centrifuged at 12,000rpm for five minutes, and re-suspended in 50ml of the MWF sample (2,000mgCOD/L, pH 7) with resazurin (1mg/L). The MWF sample inoculated with the mixed culture consisting of the COD-reducing bacteria was flushed with N_2 until the resazurin became colourless, and then kept in a shaking incubator at 35℃ at 80rpm for seven days. The relatively toxicity values (defined in section 4.3.3 in chapter 4) of the MWF sample before and after the treatment with the mixed culture were measured. Triplicate blank samples adding autoclaved biomass of the mixed culture were prepared.

The freeze-dried stock of the methanogens was re-suspended in the PBS buffer at 1Abs; 10ml of the methanogen culture was centrifuged at 12,000rpm for five minutes and re-suspended in 10ml of the treated MWF in a sealed vial (12ml). It was flushed with N_2 for five minutes and incubated in a shaking incubator at 35℃ at 80rpm for two weeks. The total volume of methane production from each sample was measured. A group of control samples containing the methanogen culture and the un-inoculated MWF sample were set up. All the samples used in this experiment were prepared with three replicates.

5.6.2 Results and Discussion

1. Assembling a Mixed Consortium Using the Selected Isolates and Optimising Its Performance on COD Reduction

Figure 5.13 below shows the relationship between the species richness of COD reducers in a mixed culture and the ecosystem functioning in terms of the COD reduction rate of the MWF. The samples for each combination were selected randomly from the pool of the COD reducer strains (detailed in section 5.5.2).

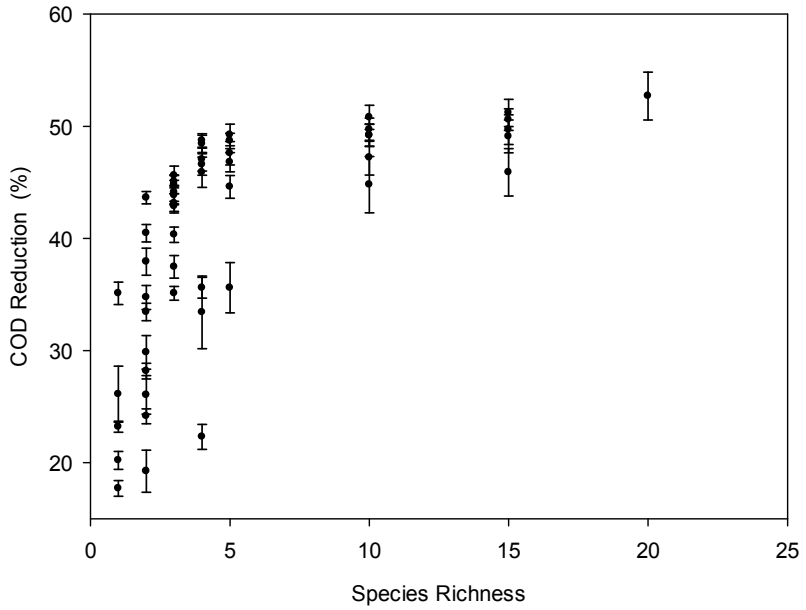

Figure 5.13: Relationship between the species richness of the COD reducers in a mixed culture and the COD reduction of the MWF over seven days (n=3, Error Bar: SD). The samples for each combination were selected randomly from the pool of the COD reducer strains (detailed in section 5.4.2)

It can be seen from Figure 5.13 that, overall, the results demonstrate a decelerating relationship between the total COD reduction and the increasing biodiversity. Bell et al. (2009) highlight two principal mechanisms employed to analyse biodiversity-ecosystem function experiments in previous studies – the "complementarity mechanism" and the "selection mechanism".

Figure 5.13 shows that the ecosystem function of the COD reducers in terms of COD reduction was more likely enhanced with increasing species richness. Firstly, the COD reducers were different species and isolated from different waste MWFs. Thus, according to the "complementarity mechanism" (Loreau and Hector, 2001; Fox, 2005; Fox and Harpole, 2008), the COD reducers could use slightly different resources; species-rich communities were therefore more efficient in using the overall resources (Bell et al.,

2009). Secondly, it has been pointed out in the "selection mechanism" that the species have variation in the magnitude of their contribution to the overall ecosystem functioning (Tilman, 1999; Loreau and Hector, 2001; Bell et al., 2005). This means the COD reducer cultures with greater species richness were more likely to include the dominant species that have relatively greater effects on the ecosystem functioning in terms of COD reduction. It was also noted that there was an upper limit to the COD reduction rate in the experiment. This limit may suggest the limit of the overall resources that could be degraded by the COD reducer species. In addition, it was suggested in Bell et al.'s study (2005), it was assumed that the various densities of the isolates in the stationary phase should have little effect on the dynamics in this experiment, which was also applied in this study.

In this part of the study, the COD reducers were employed to treat the MWF in order to overcome its toxicity. However, an anaerobic ecosystem commonly consists of various both COD-reducing bacteria and methanogens, and it has been reported that the relationship between their biodiversity and ecosystem functioning is too complicated to be investigated using current methods and approaches (Bryant, 1979; Dhaked et al., 2010). It is still a big challenge to define the mechanism underlying a clear understanding, as it requires knowledge of each species' contribution to the anaerobic ecosystem functioning, including methanogens and hydrolytic, acidogentic and acetogenic bacteria.

In addition, another important outcome of this experiment was that a standard culture was assembled for subsequent study by mixing the 20 isolated COD reducer strains equivalently.

2. Overcoming the Toxicity of RELUBRO MWF Using the Assembled Consortium

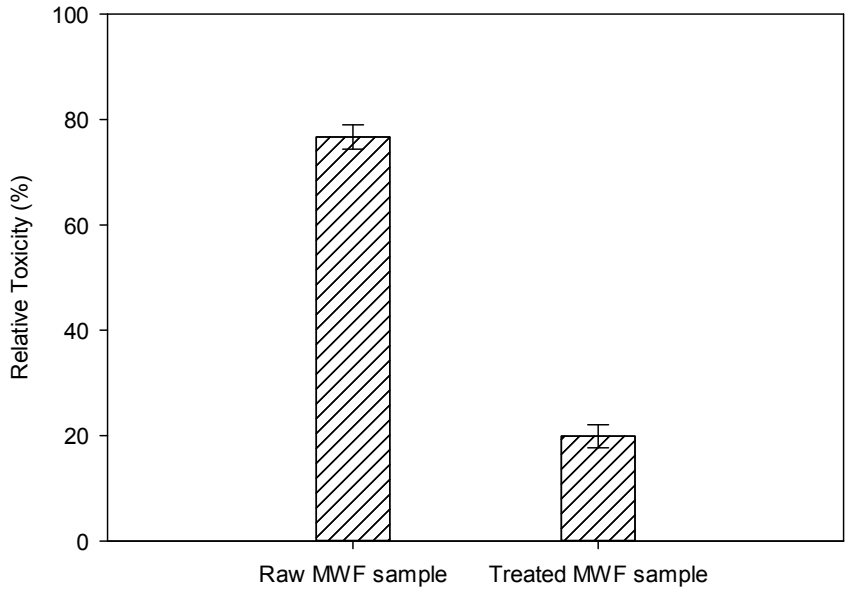

Figure 5.14: The relative toxicity reduction of the MWF treated with the mixed culture of the COD-reducing bacteria isolated from spent MWFs over seven days (n=3, Error Bar: SD)

Figure 5.14 above shows the change in the relative toxicity of the MWF (2,000mgCOD/L, pH 7) treated with a mixed consortium of the 20 exploitable COD-reducing bacteria at 35℃ over seven days (see section 5.4.2 for details regarding the COD reducers). The most striking result to emerge was that the relative toxicity of the MWF was markedly reduced (by three-quarters) over the treatment using the mixed consortium. Meanwhile, there was no change in the relative toxicity of the control samples with autoclaved mixed culture added. This noteworthy result suggests that the proposal in regard to employing the

isolated bacteria in overcoming the toxicity of MWFs and enhancing the anaerobic treatment of MWFs is a promising approach for achieving the goal.

Furthermore, around 0.9±0.2ml methane was produced from 10ml of the treated MWF sample inoculated with the mixed standard methanogen culture (DSM 7222 & DSM 4556) over two weeks, while no methane was produced from the control samples, which only contained the methanogen culture and the raw MWF sample. The methane production indicates the good activity of the standard methanogen strains in the MWF sample treated with the mixed COD-reducing consortium, which also suggests some success in terms of overcoming the toxicity of the MWF using the treatment of the COD-reducing bacteria isolated from spent MWFs.

5.7 Discussion and Conclusions

The studies described in this chapter were undertaken in order to develop a method for isolating anaerobic bacteria from spent MWFs and to evaluate the feasibility of utilising the isolates in the treatment of the MWF in order to overcome its toxic effects, thus enabling the anaerobic degradation process and methane production.

Returning to the hypothesis proposed at the beginning of this Chapter regarding employing anaerobic isolates in the treatment of MWFs, it is now possible to state that improving the treatment efficiency of MWFs by exploiting specific anaerobic bacteria species in anaerobic treatment processes is a feasible approach. The content of the studies described in this chapter revolves around two major innovations: bacteria isolation from waste MWFs and overcoming the toxicity of the MWF with the COD-reducing isolates from spent MWFs. The findings of the experiments in this chapter indicate positive outcomes for both.

The major findings to emerge from the studies described in this chapter are as follows:

I. In total, 65 COD-reducing bacteria strains were isolated from 12 samples among 20 spent MWFs samples provided by Microbial Solutions Ltd, a bio-tech company specialising in the microbiology of MWFs. Nineteen species were identified from among the 29 selected strains that had relatively greater capability of degrading the MWF. The 18 species included obligate anaerobes (such as *Desulfovibrio sp.*, *Anaerobacillus sp.* and *Clostridium sp.*) and facultative anaerobes (such as *Enterococcus sp.*, *Pseudomonas sp.* and *Escherichia sp.*), which indicates a high

level of biodiversity among the anaerobic microorganisms in waste MWFs. On the other hand, eight spent MWFs were too toxic to allow any anaerobic bacteria to grow within them, which also confirmed the significant toxicity of MWFs reported in prior studies (Rossmoore, 1981; 1983; Carvalhinha et al., 2010; Gilbert et al., 2010).

II. No pure methanogen strain was obtained from the isolation work, only two cultures isolated from waste MWFs that were composed of distinct methanogen genotypes and would only grow as colonies. Their complementary activities led to methane generation, which did not occur in the purification process. Two pure methanogen stains were purchased from DSMZ (Germany) as standard methanogen samples for further studies. The major reasons for the unsuccessful work on methanogen isolation may include: (1) their low diversity and abundance in MWFs because of the great toxicity; and (2) the demanding and meticulous growth requirements of methanogens.

III. The toxicity of the MWF was successfully reduced by the treatment employing the mixed consortium of the characterised COD-reducing bacteria isolated from spent MWFs, and methane could be generated from the treated sample by the standard methanogen strains. This finding confirms the outcomes reported by van der Gast et al. (2001; 2003a, b; 2004) regarding the feasibility of exploiting specific bacteria species to enhance the biological treatment process of waste MWFs. To our knowledge, this is the first piece of research investigating the toxic effects of MWFs on specific functional groups (particularly on methanogens) and attempt

to overcome the toxicity of compounds in MWFs by exploiting specific anaerobic strains. Taken together, the successful fulfilment of the aim of this study suggests a promising potential to recover valuable bio-energy from the huge amount of hazardous waste MWFs. In consequence. overcoming the toxic effects of MWFs on the anaerobic degradation process could result in significantly increasing the treatment efficiency of spent MWFs, and dramatically reducing the energy and financial costs relating to the disposal of spent MWFs. This research will serve as a baseline for future studies of the anaerobic degradation of the compounds in MWFs and suggest ways of developing new enhanced or hybrid treatment processes, which is similar to the Jagadevan et al.'s studies (2011, 2012). The approach of employing defined bacterial consortia that we have developed in this study is similar to the one developed by van der Gast et al. (2004) using aerobic bacteria. However, the anaerobic approach has the potential benefit of generating bio-energy from waste organic matter whilst aerobic route leads to CO_2 emission. This study had a breakthrough in overcoming the toxic effects of MWFs by exploiting specific species, and therefore enhancing the methane generation from the anaerobic degradation of MWFs. This therefore assists in further research investigating the bio-toxicity of each compound in MWFs, building on Sandin et al.'s study (1990) regarding the selective toxicity of alkanolamines, and the development of environmentally friendly MWF products, such as the employment of vegetable oils to replace mineral oils (Norrby, 2003; Matthew et al., 2007; Lawal et al., 2012). In addition, the findings in respect to the relationship between the biodiversity of the characterised COD reducers and their ecosystem

functioning in the anaerobic degradation of RELUBRO MWF has gone some way towards enhancing our empirical understanding of the anaerobic microbial systems involved in organic wastewater treatment.

In addition, there was an interesting finding in the results shown in Figure 5.13 in respect to the relationship between the species richness of the COD-reducing bacteria and their overall function in the degradation of the MWF. This provides an important way to understand the functions of the COD reducers and their interaction by employing the "complementarity mechanism" and "selected mechanism" (Bell et al., 2005; 2009). The reported studies reviewed in this study mainly focused on the theoretical studies employing some isolated bacteria strains with growth media; whilst, in this case, COD reducers isolated from spent MWF in the treatment of RELUBRO MWF provides empirical support to the theories. A future study researching the relationship between the biodiversity of an anaerobic ecosystem (including methanogens) and ecosystem functioning is highly recommended.

Chapter 6

Conclusions and Recommendations

Metalworking fluids have played a central role in modern heavy and manufacturing industries, with annual usage of MWFs exceeding 38 million metric tons worldwide (Shashidhara&Jayaram, 2010). However, when MWFs become operationally exhausted, they present a costly issue in both financial and energy terms and a great threat to public and environmental health (Calvert et al., 1998; Macherer et al., 2003; Stear, 2005). MWFs contain highly concentrated complexes of recalcitrant and toxic petroleum components; and the amount of diluted waste effluent from MWFs is more than ten times annual usage, as MWFs arrive on-site in high concentration and are then diluted prior to use (Anderson et al., 2003; Cheng et al., 2004, 2005; Shashidhara&Jayaram, 2010; Kline & Company, Inc., 2006). Thus, the disposal of spent MWFs in a sustainable way is an increasing desirable goal, particularly on-site treatment of spent MWFs is a notable goal because it can reduce the financial cost and energy consumption involved in transporting waste effluent in large volumes, and enable water to be recycled in some cases. Water security and global warming are two of the most crucial issues facing modern society. As a result, regulations relating to the saving and protection of water resources, such as the EC Water Framework Directive 2000, UK River Basin Management Plans (2006), UK Integrated Pollution Prevention and Control Directive, UK Environmental Permitting Regulations, and UK Water Act 2003, become increasingly stringent. Moreover, demand for energy savings and reduced carbon emissions through industry are also increasing

across the world. The development of new methods for the sustainable disposal of spent MWFs is driven by both the tightening of governmental regulations and urgent demands in industry. The goal of this study was to employ an anaerobic degradation process in the disposal of spent MWFs resulting in the removal of the organic pollutants from the water and the recovery of organic components in the form of bio-energy by the generation of biogas. However, the major challenge of this study was to reduce the toxic effects of MWFs on the anaerobic microorganisms involved in the degradation process, especially on methanogens, a functional group that is very sensitive to extreme growth environments and toxic matter.

In general, the disposal methods of spent MWFs can be classified into three categories — chemical, physical and biological methods (Cheng et al., 2005). The chemical treatment methods applied in the disposal of spent MWFs include de-emulsification with chemicals, electro-coagulation and advanced oxidation processes (Rios et al., 1998; Cheng et al., 2005; Jagadevan et al., 2012). Physical treatment methods employed in the disposal of MWFs mainly include evaporation, membrane filtration, microfiltration, ultrafiltration, nanofiltration, reverse osmosis (RO) and peat adsorption (Rios et al., 1998; Skerlos et al., 2000; Hilal et al., 2004; Cheng et al., 2005). In the UK market, membrane filtration is popular in the disposal of MWFs due to their high level of efficiency (over 90%) in terms of separating petroleum compounds from wastewater (UKLA, 2011). However, it also has noticeable disadvantages in terms of both energy demand and technical limitations. Membrane filtration requires a large amount of energy consumption during the treatment process; and membrane fouling results in the treatment efficiency being rapidly reduced, which also entail a heavy cost on replacement (Benito et al., 2000; Ezzati et al.,

2005;Hesampour, et al. 2008). In particular, petroleum compounds are only concentrated in physical treatment processes; no appropriate disposal method for the highly concentrated pollutants has emerged, since the landfills and the incineration of hazardous wastes are forbidden in the UK.

Compared with chemical and physical methods, biological treatment methods, including aerobic and anaerobic processes, have significant advantages, such as their low capital, operating and maintenance costs, plus the fact that no hazardous chemicals are required (Kim et al., 1994; van der Gast et al., 2003; Rabenstein et al., 2009; UKLA, 2011). Thus, the biological treatment of spent MWFs is an increasingly attractive option, particularly anaerobic treatment method, which also has the potential benefits of energy saving and bio-energy generation from organic wastewater. Compared with aerobic degradation process, anaerobic degradation process enables us to convert the organic matter in wastewater into biogas instead of consuming an enormous amount of energy while also producing immense CO_2 emissions (Fang, et al., 1996; Shink, 2002). The complex compositions and toxicity of MWFs represent the major challenges in terms of employing anaerobic treatment method in the disposal of MWFs. Over 300 chemical constituents are known to be employed in MWFs including organic and inorganic components (Rabenstein et al., 2009). MWFs mainly contain base oil, emulsifiers (surfactants), corrosion inhibitors, extreme pressure agents, biocides, and alkaline reserve agents (Anderson et al., 2003). The combination of the compounds in high concentration either results in great toxicity to any microorganisms that come into contact with them or inhibits their growth and activity (Cheng et al., 2005; Carvalhinha et al., 2010; Gilbert et al., 2010). Furthermore, anaerobic microorganisms are more sensitive to toxic

environments; in particular, methanogens are very sensitive to changes of temperature and pH, as well as to toxic compounds. RELUBRO 800 BIO (MacDermind plc, Birmingham, UK), a typical water-based MWF, was used in this study investigating the anaerobic treatment of MWFs.

To find the solutions needed to overcome the toxic effects of the semi-synthetic MWF on its anaerobic degradation process and to stimulate the generation of methane, this study was divided into three major phases. Firstly, the anaerobic biodegradability of RELUBRO MWF was investigated in experimental systems inoculated with anaerobic activated sludge as an initial investigation regarding the toxic effects of the MWF on its anaerobic degradation process, particularly on the methane production of that process. Secondly, a further study examining the toxic effects of the MWF on methanogen species was carried out to determine the specific impacts of the toxic effects of the MWF and the feasibility of overcoming the toxicity. Thirdly, based on the outcomes of the first two phases of this study, a toxicity-overcoming approach was developed by re-inoculating the anaerobic bacteria isolated from waste MWFs back into the treatment process.

6.1 Anaerobic Degradation of the MWF

Anaerobic treatment approach has been widely employed for the treatment of various industrial organic wastewaters; however, this approach has received very little attention in relation to the treatment of spent MWFs (Lettinga, 1996; Shink, 2002, Cheng et al. 2005). As was touched on above, the major challenges in terms of employing anaerobic treatment process in the disposal of spent MWFs can be summarised as three points: 1) the complex compositions of MWFs; 2) the toxicity of MWFs; and 3) the sensitivity of

anaerobic microorganisms to toxic environments.

A typical water-based MWF (RELUBRO 800 BIO, MacDermind plc) was used in this study. As the first phase of this study, the anaerobic biodegradability of the MWF was investigated in an experimental system inoculated with anaerobic activated sludge. The COD concentration of the MWF samples taken in this study was around 100,000mg/L. However, the BOD/COD ratio was around 0.34, which indicates a low level of biodegradability. The MWF contains alkaline reserve agents and biocides, and they result in an environment that is toxic to microorganisms, with a high pH value (8.7). The high concentration, low biodegradability, high pH value and existing biocides in RELUBRO MWF were the major challenges in terms of employing anaerobic treatment approach in this study.

Two of the most important findings to emerge from this study are that approximately 80% of RELUBRO MWF at 5,000mgCOD/L was anaerobically biodegradable, and that approximately 35% of the biodegraded COD could be converted to methane in the experimental system inoculated with anaerobic activated sludge. There are similarities between these findings in this study and those described by Kim et al. (1989; 1990; 1992). They carried out a comprehensive study on the anaerobic treatment of MWFs using a GAC fluidised-bed reactor. In their work, approximately 65% of the influent organics in a simulated MWF was found to be anaerobically biodegradable, and approximately 68% of the biodegraded COD was converted to methane. However, the composition of the simulated MWF was not reported in their studies.

RELUBRO MWF also appeared to play an inhibitory role in regard to anaerobic

microorganisms, especially methanogens — the COD reduction and methanogen production rates decreased with increasing concentration of the MWF. This is not surprising because MWFs are specifically formulated so that they are resistant to microbial colonisation and bio-deterioration while in use in machines. This finding supports previous research into this area which links the low biodegradability of MWFs and their toxic effects on microorganisms (Kim et al., 1989; 1990; Perez et al., 2006; van der Gast et al., 2004; Muszynski & Lebkowska, 2005; Cheng et al., 2005). Therefore, it can be concluded that the biodegradability of a MWF depends on its composition and characteristics, especially its toxicity to the microorganisms involved in the treatment processes.

Besides of the intrinsic resistance to biodegradation of the petroleum compounds in MWFs, the literature review reveals that the toxicity of MWFs is the biggest challenge facing in the biological disposal of spent MWFs, regardless of whether aerobic or anaerobic processes are employed (van der Gast et al., 2004; Cheng et al., 2005). The toxicity of MWFs referred to in reported studies generally consists of three parts, including the presence of biocides, the toxicity of degradation products from the substances in MWFs, and unfavourable environmental factors (pH and temperature) (Rossmoore, 1991; Gilbert et al., 2010). Aerobic bacteria are normally considered as having relatively greater capability to tolerate toxic compounds and unfavourable environmental conditions including high pH and toxic matters. However, conventional aerobic sludge systems are not suitable for the disposal of highly concentrated organic wastewater because of the high energy consumption involved and the production of a large amount of biomass, and they do not have the benefit of generating energy from

waste (Backer et al., 1983; Cheng et al. 2005). Thus, several studies have attempted to employ isolated aerobic bacteria in the treatment of MWFs (van der Gast et al., 2004; Muszynski & Lebkowska, 2005; Connolly et al., 2006 & Bakalova et al., 2007). Most of them reported good removal efficiencies (60—90%) in regard to the organic pollutants in their MWF samples; however, these studies were carried out within limited experimental conditions, such as a low concentration of MWF samples and additional supplement of nutrients, or no characterisation of the MWF samples used in their study (Kim et al., 1992a, 1994; Deepak et al., 1994, Schreyer and Coughlin, 1999; van der Gast et al., 2003). Compared with aerobic treatment process, the anaerobic treatment process is much more complicated in terms of microbial community structure and metabolic pathway (Scragg, 1999; Appels et al., 2008). In Bryant's theory (1979), the anaerobic degradation process consists of various biological conversion steps producing intermediate metabolite products, and each step is performed by a specific type of anaerobic microorganism. It has two major disadvantages affecting its employment in the disposal of MWFs: 1) Anaerobic microorganisms are more sensitive to toxic matters, especially methanogens; and 2) the growth rates of anaerobic microorganisms are quite low (Fang et al., 1996; Shink, 2002; Cheng et al., 2005). In this study, the anaerobic biodegradability of RELUBRO MWF was investigated in an experimental system inoculated with anaerobic sludge. The outcome of the investigation indicated that, compared with the control group, the low biodegradability and toxic effects of the MWF resulted in a 10–16% drop of COD reduction rates and in a 60– 70% reduction in the methane production yields. The inhibitory effect of the MWF on methane production can also be seen in that methanogens are more sensitive to toxic environment, and

methanogens are the key to converting organic matters into bio-energy in form of methane (Bryant, 1979; Dhaked et al., 2010). Additionally, the toxicity of the MWF was not lethal to all of the anaerobic bacteria in the experimental system inoculated with anaerobic sludge, such as the methanogens in the sludge, which suggests there was great potential for enhancing the anaerobic microbial community to convert the organic wastes in MWFs to methane.

There are several kinds of microorganisms involved in this process; in most of the prior studies, they were categorised according to their ecosystem function, such as hydrolytic, acid-forming bacteria (acidogentic and acetogenic bacteria) and methanogens (Bryant, 1979; Lettinga et al., 1980; Hulshoff Pol et al., 2004; Scherr et al., 2012; Singh et al., 2012). In this study, the anaerobic bacteria were divided into two main categories according to their functional roles in the anaerobic degradation processes: COD-reducing bacteria and methanogens. The COD reducers consisted of hydrolytic, acidogenic and acetogenic bacteria that degrade the organic compounds within the MWFs into small-molecule fractions which are subsequently assimilated and converted to biogas by the methanogens. The anaerobes work as a community whereas a single aerobic strains can be solo colonised in a toxic chemical; and methanogens have been shown to be very sensitive to unfavourable temperature, pH and toxicity environments, especially methanogens are solo colonised (Dhaked et al., 2010; Patidar et al., 2005; Chen et al., 2007; Hoj et al., 2008). Moreover, the methane production from RELUBRO MWF indicates the existence and persistence of methanogens within a community such as anaerobic sludge, which also suggests the feasibility of enhancing methane production in the degradation process of the MWF by exploiting a microbial system instead of an

individual strain. Therefore, a comprehensive study examining the toxic effects of the MWF on methanogenesis was carried out as the second phase of this study.

6.2 Examining the Toxic Effects of the MWF on Methanogenesis

The toxicity of MWFs on exposed microorganisms has often been highlighted in prior studies attempting to exploit biological systems in the disposal of MWFs, including employing aerobic and anaerobic routes (van der Gast et al., 2001; 2004; Perez et al., 2006). There were few successful cases attempting anaerobic treatment of MWFs because of the toxicity of MWFs and the sensitivity of anaerobic bacteria to toxic environment (Kim et al., 1992; Cheng et al., 2005). This study is the first of its kind to investigate the toxic effects of MWFs on one of the most important anaerobic bacteria species – methanogens. It is well known that methanogens are the key to methane production in anaerobic degradation process and the most sensitive species in the microbial community to a toxic environment. Overall, the results of this part of study revealed the significant toxic effects of the MWF in regard to reducing the activity of methanogens and altering the community structure of methanogens.

The prior studies suggest that there are several factors affecting the methanogens in the anaerobic degradation process. These include pH value, temperature, the inoculum community structure, the composition of substrate and toxic compounds (Koster & Lettinga, 1984; van Haandel & Lettinga, 1994; Colleran et al., 1998; Sun et al., 2000; Chen et al., 2007;). pH has direct effects on the methane yield. In this case, the high pH of MWFs (pH >8) helps biocide activity (Sandin et al., 1990,1991). Sandin et al. (1991) reported that the antimicrobial activity of butylethanolamine and related compounds

increased at a higher pH (over 9), which made these compounds suitable for microbial control in MWFs. However, the pH range for optimising methanogenic activity in anaerobic treatment process is between 6.3 and 7.8 (Anderson & Yang, 1992; van Haandel & Lettinga, 1994; Lettinga et al., 1996). The results of this study suggest a specific pH range for exploiting isolated COD reducers and methanogens was in the range between 6.5 and 7. Temperature is also an important factor affecting anaerobic degradation processes. Previous studies suggest that it has effects both on the activity and the biodiversity of anaerobic bacteria (Anderson & Yang, 1992; El-Mashad et al., 2004; Xie & Liu, 2007; Lone, et al., 2008). The findings regarding the effect of temperature indicate that, in general, the bacterial activity levels of anaerobic bacteria incubating at ambient temperatures (18℃～30℃) were less than those at mesophilic (30℃～40℃) and thermophilic (50℃～60℃) temperatures, and it had effects in terms of altering the community structure of anaerobic microorganisms (Anderson & Yang, 1992; Xie & Liu, 2007; Lone et al., 2008).

Besides these environmental factors, the presence of ammonia, sulphide, heavy metal, and biocides can also inhibit the activity and biodiversity of methanogens in an anaerobic degradation system (Harada et al., 1994; Whittmann et al., 1995; Chen et al., 2007, Cheng et al., 2005). In particular, the presence of biocides in MWFs, such as metallic, phenolic or chlorinated organic matter and formaldehyde (FA)-based industrial biocide and organosulphur nitrogen compounds, aims to inhibit and control the growth of microorganisms (Anderson et al., 2003; Gilbert et al., 2010). It has been reported that, methanogens are relatively more sensitive to ammonia and sulphide inhibition compared

with other anaerobic bacteria (Koster & Lettinga, 1984; Kayhanian, 1994; Colleran et al., 1998). Heavy metals are not biodegradable and can be accumulated in microorganisms to toxic concentration, while also causing disruption of enzyme function and structure in microbes (Vallee & Ulner, 1972; Mori et al., 2000). The toxicity of RELUBRO MWF caused by the inhibitors in its composition was defined and investigated as one factor (relative toxicity). This is because its formulation details are only partially divulged by the manufacturer due to their commercial sensitivity, meaning there was no way to determine all the compounds that could potentially affect the activity of methanogens and investigate them individually.

The research approaches in most of the prior studies of the anaerobic treatment of MWFs only focused on two general indicators, specifically the COD reduction and methanogen production rates (Kim et al., 1989, 1990, 1992, 1994; Perez et al., 2006; 2007). Theoretically, these two indicators are able to describe the general status of an anaerobic degradation process in terms of treatment efficiency and specific methanogenic acivity (SMA) — organic transformation rate. However, these two indicators are not by themselves capable of characterising the overall toxicity of an MWF on an anaerobic degradation process and its bacteria community. Thus, changes to the anaerobic bacteria community in the degradation of MWFs have received very little attention (Scherr et al., 2012). Two molecular profiling methods (FISH and DGGE) greatly aided the characterisation of the toxic effects of RELUBRO MWF on the diversity and community structure of methanogen communities in the anaerobic degradation process, as these molecular biological techniques enable detection of many microbial species without isolation or cultivation of microorganism. In this study, the results of the experiments

employing FISH probes indicate that there was an unapparent change in the overall biomass of methanogens in the sludge samples fed with the MWF over 20 days, whereas the DGGE results show some methanogen bands corresponding to certain methanogen species having disappeared during the 20-day operation. These FISH and DGGE results suggest the MWF seems very toxic to specific populations within the methanogen community, which also suggest the possibility of employing those methanogen species with less sensitivity to the MWF or overcoming the toxic effects so as to improve the methane production rate in anaerobic degradation process of the MWF. The results of the FISH analysis also indicated that there was no significant change to the biomass of COD reducers and methanogens in the sludge used in the treatment of RELUBRO MWF, which supports the aforementioned proposal regarding overcoming the toxicity of the MWF by isolating and cultivating specific anaerobic bacteria strains colonising spent MWFs and then re-inoculating them back into the treatment system. However, the COD reducers in the sludge samples consisted of various species with regards different functional roles such as hydrolysis, acidogenesis, and acetogenesis (Lettinga et al., 1980; Appels et al., 2008). It was therefore impossible to characterise the functional activity of each individual species in the anaerobic degradation process of various constituents in MWFs. This suggests that further studies should be carried out with specific strains of COD-reducing bacteria so as to obtain a clear understanding of their ecosystem functions in terms of decomposing the toxic compounds and therefore reducing toxicity of the MWF. However, because of their inherent limitations, the integrity of the information regarding microbial community structures can hardly be revealed by these methods. Some second-generation DNA sequencing platforms, such as 454-pyrosequencing and

Illumina MiSeq, have gained increasing attention as novel tools for investigating the diversity and abundance of microorganisms in bacterial communities with greater integrity and accuracy (Ye et al., 2011; Liao et al., 2013; Shen et al., 2013; Lee et al., 2014). These new methods greatly aid the characterisation of the microbial community structure involved in biological wastewater treatment processes (Hu, M. et al., 2012). However, these second-generation high-throughput sequencing methods are too costly to have been employed in this study.

In addition, an analytical method based on a bioluminescence-based bacterial biosensor, *E.coli HB101_pUCD607_lux* (Mwinyihija, 2011), was developed in order to carry out the real-time evaluation of the toxic effects of MWFs. Based on the toxicity analytical method, a single factor – Relative Toxicity – was defined to indicate the overall toxic effects of MWFs on the anaerobic degradation process.

To sum up, the major findings in the first two phases of this study suggest a promising approach to overcome the toxicity of the RELUBRO MWF by exploiting specific anaerobic bacteria species which had previously adapted to the toxic conditions of MWFs in the treatment process.

6.3 Overcoming the Toxic Effects of the MWF on Anaerobic Degradation Process

The toxicity of MWFs can be defined as the degree to which MWFs' compounds can inhibit the growth and activity of the anaerobic microorganisms involved in the anaerobic degradation process. In this study, the sensitivity of anaerobic bacteria to the toxic environment in RELUBRO MWF resulted in inhibition in terms of degradation of the efficiency of COD reduction and methane production rates. It thus raised the possibility

of overcoming the toxicity of the RELUBRO MWF by exploiting specific anaerobic bacteria species in the treatment process, which had previously adapted to the toxic conditions of MWFs and that could function to reduce the toxicity of MWFs.

The rationale was to isolate and cultivate the exploitable anaerobic bacteria species colonising spent MWFs, select and characterise the isolates, and then re-inoculate them into the treatment of the MWF in order to overcome the toxicity of the MWF. To our knowledge, this part of the study is the first research investigating the functional activity of specific anaerobic strains in terms of degrading the toxic compounds in MWFs. Undoubtedly, the implementation of this approach is challenging and difficult. The streak plate method was employed under anaerobic conditions (i.e. an anaerobic indicator, anaerobic jar, anaerobic atmosphere bag, and redox potential reducers) to isolate anaerobic bacteria from spent MWFs. This was chosen instead of the Hungate Tube method (Sharma, 2007, p. 173), which is much more difficult to undertake under experimental conditions. The employment of two bio-molecular analytical techniques (PCR and DNA sequencing) successfully increased the likelihood of isolating anaerobic bacteria by efficiently identifying different species. Theoretically, without the aid of the bio-molecular analytical techniques, less than 1% of the anaerobic bacteria species present in such an environment could be isolated and identified using traditional culture-based processes (Amann et al., 1995). In particular, with the bio-molecular methods, it was found out that the failure to isolate methanogens was mainly due to the fact that they were rarely present in spent MWFs.

Although the bio-molecular aided approach provided a relatively greater likelihood of the

isolation process being successful and much greater efficiency in terms of the identification of the isolates; it was still based on traditional culture-based or culture-independent process with the limitations of isolation efficiency in terms of a time-consuming enrichment process and extremely low possibility of isolating pure methanogen strains. Firstly, this isolation approach is time-consuming because the growth rates of anaerobic bacteria are much lower than aerobic bacteria (Chen et al., 2007), and repeating the culture based purification procedure to obtain pure strains took as long as several months. Secondly, the isolation approach employed was unable to increase the possibility of isolating pure methanogen strains from spent MWF, a habitat that is less likely to favour the persistence of methanogens. No pure methanogen strains were isolated from spent MWFs, but rather two mixed cultures containing methanogens were obtained. The existence of methanogens in the two cultures was confirmed using the 16s rRNA FISH probe. However, the attempt to purify the methanogen strains in the cultures using the bacteria-isolation approach failed. Besides the issue of methanogens having great sensitivity to toxic environments, the other reason for the failure to isolate methanogens may be that they are obligate anaerobic bacteria and most of them are individually unculturable (Hungate, 1950; Xun et al. 1990; Sheppard et al., 2005; Ariesyady et al., 2007; Singh et al., 2012). Ultimately, there is as yet no truly efficient method to isolate and purify the anaerobic bacteria present in environments.

In this study, anaerobic bacteria were divided into two main categories according to their functional roles in the anaerobic degradation processes – COD-reducing bacteria and methanogens. The COD-reducing bacteria were the anaerobic bacteria that degraded the organic compounds within the MWFs into small-molecule fractions, which could be

subsequently assimilated and converted to biogas by the methanogens. The study has conclusively shown that the biodiversity and community structure of an anaerobic ecosystem have impacts on its functionality in terms of the degradation of substrates, in this case the organic components of MWFs. In particular, the key to overcoming the toxic effects of MWFs on the anaerobic degradation process was to maximise the functional activity of COD-reducing bacteria in terms of degrading the toxic organic compounds in MWFs.

Previous research findings in anaerobic microbiology demonstrate the great complexity of anaerobic habitats, especially their interrelationships and interactions; and, according to two principal mechanisms employed to analyse biodiversity-ecosystem function experiments in previous studies – the "complementarity mechanism" and the "selection mechanism" – greater microbial diversity should theoretically give greater functional diversity in a study such as this, aiming to maximise the functional activity of an anaerobic ecosystem (Bryant, 1979; Loreau and Hector, 2001; Fox and Harpole, 2008; Bell et al., 2005, 2009). Nevertheless, contemporary understanding of anaerobic ecosystem still remains weak (Appels et al., 2008). Because of this limited understanding of anaerobic microbiology, this part of the study involving the assembling of a mixed consortium and maximising its performance in terms of overcoming the toxicity of RELUBRO MWF had to be carried out with limited experimental conditions in terms of focusing on only one variable – biodiversity (i.e. the number of COD reducer species). Even though only 20 species of COD reducers were exploited in assembling the mixed consortium, there was no way to experiment with all possible combinations of them at various proportions of biomass to obtain a true understanding of the ecosystem in the

limited time available to this study. This is because such an understanding requires knowledge of each species' contribution to the ecosystem functioning as well as the changes in the ecosystem during the experimental period. In fact, an anaerobic ecosystem, such as the anaerobic sludge used in this study, may contain hundreds and thousands of different species, including COD-reducers and methanogens. There was an interesting finding in the results in respect to the relationship between the species richness of the exploitable COD reducers and their overall function in the degradation of the MWF. The prior studies reviewed here were mainly the theoretical studies employing some isolated bacteria strains with growth media, such as the "complementarity mechanism" and "selected mechanism" (Bell et al., 2005; 2009); in this case, however, the fact that the COD reducers were isolated from spent MWF in the treatment of RELUBRO MWF provides empirical support to the theories.

Eventually, the toxic effects of the RELUBRO MWF were successfully reduced by the treatment employing the mixed consortium of the characterised COD-reducing bacteria isolated from spent MWFs, and methane was generated from the treated sample using the standard methanogen strains. This finding, while preliminary, suggests some success in terms of achieving the goal in the proposal in regard to employing exploitable anaerobic species for overcoming the toxicity of MWFs and enhancing the anaerobic treatment of MWFs. It also confirms the outcomes reported by van der Gast et al. (2001; 2003a, b; 2004) regarding the feasibility of exploiting specific bacteria species to enhance the biological treatment process of waste MWFs.

6.4 Future Work and Recommendations

This study has revealed a promising potential to recover valuable bio-energy from the huge amount of hazardous waste MWFs, while also raising several questions in need of further investigation in future work.

First of all, the aim in regard to overcoming the toxicity of RELUBRO MWF by exploiting the anaerobic isolates obtained from waste MWFs was successfully fulfilled; employing this approach to enhance the anaerobic treatment of waste MWFs seems to be quite a promising avenue. Thus, a future study investigating the feasible ways to employ the findings in the engineering development of new enhanced or hybrid treatment processes for waste MWFs would be very interesting. For example, the aerobic treatment method developed by van der Gast et al. (2003) or the nano-zerovalent iron oxidation method developed by Jagadevan, et al. (2012) could be employed with the anaerobic degradation process, thus exploiting the isolated anaerobic bacteria as pre-treatment steps for further overcoming the toxicity and therefore enhancing the bio-energy recovery in the anaerobic treatment process. However, the aerobic and chemical oxidation could also reduce the carbon sources for bio-energy generation, and this type of hybrid treatment process thus requires further studies in regard to their feasibility and optimisation. The success of this development could result in significantly increasing the treatment efficiency of spent MWFs, dramatically reducing the energy and financial costs relating to the disposal of spent MWFs, and enhancing bio-energy recovery from the disposal process instead of producing CO_2 emissions. However, more challenges arise when it comes to the process of developing such a new treatment process, including the low

growth rate of anaerobic bacteria and the difficulty of immobilising the anaerobic bacteria in a bio-reactor. Moreover, if the isolated bacteria are combined with the activated sludge processes, then it would be necessary to develop a research method for evaluating and quantifying the performance of exploiting anaerobic isolates in an complex anaerobic ecosystem consisting of various different species.

Secondly, the major components of the MWF used in this study are also widely used in most MWFs, such as refined mineral oil and organic solvents. The findings of this study highlight that most of these compounds are anaerobically biodegradable, and the MWFs, as mixtures of them, could have seriously toxic effects on the anaerobic degradation process and significant toxicity for methanogen species. Consequently, further research might investigate the toxicity of each compound and the interactions of their anaerobic degradation processes. For example, Sandin et al. (1990) carried out a study regarding the selective toxicity of a few different alkanolamines (diethanolamine, dimethylamino-methyl-propanol, and butylethanolamine) on a strain of *Pseudomonas pseudoalcaligenes* isolated from used metalworking fluids, and their results showed that the toxicity of the alkanolamines was greatly enhanced at high pH. The same species was also isolated from the spent MWF samples in this study. This could also be a reasonable approach through which to understand the key toxic compounds and to look for replacements with low toxicity, such as using vegetable oils to replace mineral oils and the like (Norrby, 2003; Matthew et al., 2007; Lawal et al., 2012). There is, therefore, a definite need to develop a new real-time toxicity analytical method that has a higher upper limit of sample concentration and less variation in terms of changing pH.

Thirdly, although this study could be the beginning of anaerobic isolates being employed in the disposal of MWFs, the bacteria isolation process employed in this study was time-consuming and had low efficiency in terms of number of exploitable strains it yielded. To date, there has been no efficient method of isolating a single pure strain of anaerobic bacteria from a mixed colony directly. It is thus recommended that further research should also focus on developing non-culture-based isolation approaches for anaerobic bacteria with the aid of the development of bio-molecular analytical and experimental technologies. In addition, further studies in regard to the anaerobic degradation of each component of MWFs could be quite helpful in terms of increasing our understanding of the functions of each bacteria species. It would also be very interesting to find out more about the interaction between the different anaerobic bacteria species in an ecosystem.

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APPENDICES

Appendix 1: Formula of Nutrient Broth

Formula of CM0001 Nutrient Broth (Oxoid, UK) include: 'lab-lemco' powder 1.0g/L; yeast extract 2.0g/L; peptone 5.0g/L and sodium chloride 5.0g/L. Its pH is 7.4±0.2 at 25℃.

Appendix 2: Procedure of Fluorescence *In-Situ* Hybridisation Probe Test

The experimental protocol of FISH test was amended and adapted from the protocol provided by Molecular Probes, Inc. (Life Technologies Corporation).

(1) Materials and Reagents

FISH probe for methanogens; FISH universal probe; 1×PBS buffer solution; 3×PBS buffer solution; 4% Paraformaldehyde (PFA) solution; Hybridisation Cleaning Solution: 9.9g NaCl, 20ml Tris-HCl solution (1mol/L), 980ml Milli-Q water; Hybridisation Buffer Solution: 0.01% sodium dodecyl benzene sulfonate (SDBS), 20 mM Tris-HCl, 20% formamide, and 0.9M NaCl.

(2) Experimental Procedure

1) Add 300 µl sludge sample and 900 µl 4% PFA solution into 1.5ml eppendorf tube and mix the mixture until homogenous. Then keep the mixture at 4℃ overnight.
2) Centrifuge the sludge sample mixture at 12,000rpm for 5 minutes and discard the top liquid layer and wash the sludge sample with 1×PBS buffer solution three

times.

3) Re-suspend the sludge sample in 1ml 1×PBS buffer solution.
4) Warm specimen slide to room temperature and pre-warm hybridisation buffer at 73℃ for 30 minutes.
5) Immerse slides in hybridisation buffer at 73℃ for 5 minutes.
6) Singly place slides in 70%, 85% and 100% ethanol at room temperature for 3 minutes.
7) Gently dry back of slide and place on slide warmer at 30℃ until ethanol evaporates.
8) Add 100µl sludge sample to slide and let dry in dark.
9) Add 10µl probe mixture (2µl probe and 8µl hybridisation Buffer Solution) on the dry sludge sample, then apply clean coverslip to slide and incubate the sample at 46℃ for 6 hours.
10) Clean the slides and visualise under microscope using appropriate filter sets.

The images of FISH test were analysed using Motic Fluo Software (Motic Incorporation Ltd. Hong Kong).

Appendix 3: Procedure of Denaturing Gradient Gel Electrophoresis

DGGE was performed with a Dcode Universal Mutation Detection System (Bio-rad, UK) according to the instruction provided by the manufacturer.

(1) Materials and Reagents

a) PCR primers

b) Tetramethylethylenediamine (TEMED)

c) 50×Tris-Acetate-EDTA (TAE) buffer

d) 40% Acrylimide Solution: Dissolve 38.93g Acrylimide and 1.07g Bis-Acrylimide in 100ml Milli-Q water, and filter the mixture solution with 0.45μm syringe filter and keep at 4℃.

e) 10% Ammonium Persulfate (APS): Dissolve 1g APS in 10ml Milli-Q water and keep at 4℃.

f) 30% Denaturing Reagent: dissolve 20ml 40% Acrylimide Solution, 2ml 50×TAE buffer, 12ml Formamide, 12.6g Urea in Milli-Q water and make up to 100ml. Filter the mixture solution with 0.45μm syringe filter and keep at 4℃.

g) 40%, 60% and 70% Denaturing Reagents.

h) Staining Reagent: dissolve 0.05g Bromophenol Blue and 0.05g Xylene Cyanol FF in 10ml TAE buffer, and keep at -20℃.

i) DGGE Kit (*Bio-Rad*) and GelDocXR gel documentation system (Bio-Rad).

(2) Experimental Procedure

① Clean and dry the glass gel casting plates thoroughly with detergent, DI water and ethanol.

② Add 25μl TEMED and 110μl 10% APS to the denaturing reagents and immediately stir the mixture.

③ Transfer the mixed gel into syringes and pump the gels into the DGGE gel caster.

④ Place the comb in the gel and leave the gel at 37℃ for 1.5 hours.

⑤ After the gel has polymerised, remove the comb, place the gel in the gel running

cell and fill the tank with TAE buffer, heat to 60℃.

⑥ Load 20µl PCR products with 20µl gel loading buffer, then turn on the power supply to start to run the gel.

⑦ The images of the DGGE were taken using GelDocXR gel documentation system (Bio-Rad).

Appendix 4: Genomic DNA Extraction Protocol

In this study, the genomic DNA of the anaerobic bacteria was extracted using the following protocol for further genetic fingerprint analysis (Green and Sambrook, 2012, page 1-78).

a) Collect 15mg of cells from an agar plate and put into a 1.5ml eppendorf tube (micro-centrifuge tube).
b) Add 400µl of TE buffer (100mmol/L Tris, 100mmol/L EDTA, pH 8.0) into the eppendorf tube for disperse the bacterial pellet, and mix the cell pellet with TE buffer using vortex mixer twice for 20 sec each time.
c) Add 50µl of 10% SDS, incubate the bacteria lysate at 65℃ for 0.5-3 h in water bath (Optimisation of the splitting time is required). Then add 50µl of proteinase K (20mg/L in TE, pH 7.5), and incubate at 55℃ for 1 h in water bath.
d) Add 100µl 5mol/L NaCl solution and 80µl CTAB/NaCl mixture (10mol/L CTAB, 0.7mol/L NaCl) into the eppendorf tube, and incubate at 65℃ for 10 minutes in water bath.
e) Add 500µl mixture of isoamly alcohol: chloroform (1:24) into the eppendorf tube.

Shake well to form an emulsion but do not vortex. Spin the tube in the centrifuge for 5 minutes at 12,000r/min.

f) Remove the top aqueous layer into an eppendorf tube, and add equal volume of isopropanol. Mix carefully by inverting the tube, and leave the DNA to completely precipitate out by leaving at room temperature for 10 min and store at -20 ℃ overnight.

g) Spin the tube at 12,000r/min for 20 minutes to pellet the DNA, pour off the supernatant gently and place the tube upside down on some brow paper towel to remove excess isopropanol.

h) Wash the DNA pellets with 100µl 80% ethanol, spin the tube at 12,000r/min for 5 min, and repeat this step twice.

i) Pour off the supernatant gently and place the tube upside down on some brow paper towel to removal excess ethanol.

j) Place the tube in a rack to allow the pellet to air dry before re-suspending.

k) Re-suspend the DNA in 200µl of TE buffer with 1µl RNAse. Incubate the solution for 30 minutes at 35℃.

l) DNA extracted is stored at -20℃.

The quality of the extracted DNA was evaluated using agarose gel electrophoresis.

Appendix 5: PCR Amplification Procedure

According to the recommended PCR amplification procedure of Sigma-Aldrich and the procedure reviewed by Scherr. K. E. et al. (2012) and Green & Sambrook (2012, page

455-681), the PCR amplification procedure applied in this study is designed and developed as follows.

(1) PCR reagents

PCR buffer (BIOLINE, Fisher Scientific); Deoxynucleotide (dNTP) mix, 10mM (Sigma-Aldrich); $MgCl_2$ solution, 50mM (BIOLINE, Fisher Scientific); Taq DNA polymerase (Sigma-Aldrich); PCR Primers (Invitrogen, Life Technologies).

50µl premix solution was applied for each PCR reaction, including: 5µl PCR buffer, 1µl dNTP, 1µl -5µl DNA template, 1µl forward primer, 1µl reverse primer, 1µl Taq DNA polymerase and PCR water.

(2) PCR machine and reaction programme

PCR machine used in this study was The PCR machine used in this study was Bio-Rad MJ Mini Personal Thermal Cycler. The PCR reaction programme is given as follows:

Table A. 1: PCR reaction programme applied for PCR amplification

Cycle number	Denaturation	Annealing	Polymerization
35 cycles	30 sec at 94 ℃	30 sec at 55 ℃	1 min at 72 ℃
Last cycle	1 min at 94 ℃	30 sec at 55 ℃	1 min at 72 ℃

The PCR amplification products were extracted from agarose gel using a gel extraction kit (GenElute, Sigma-Aldrich).

Appendix 6: Media and Stock Solutions for Bacteria Isolation

All the formulation details of the media and stock solutions are given as follows; they were adapted or developed from the formulations provided by DSMZ and Dr. Andy Lilley.

① Trace element solution

The formula of the trace element solution used in this study was provided by DSMZ (Germany). It includes: $MgSO_4 \cdot 7H_2O$ (3g/L), $MnSO_4 \cdot H_2O$ (0.5g/L), NaCl (1g/L), $FeSO_4 \cdot 7H_2O$ (0.1g/L), $CoCl_2 \cdot 6H_2O$ (0.18g/L), $CaCl_2$ (0.1g/L), $ZnSO_4 \cdot 7H_2O$ (0.18g/L), $CuSO_4 \cdot 5H_2O$ (10mg/L), $AlK(SO_4)_2 \cdot 12H_2O$ (20mg/L), H_3BO_3 (10mg/L), $NaMoO_4$ (10mg/L), $NiSO_4 \cdot 6H_2O$ (30mg/L), $NaSeO_3$ (0.3mg/L), , Nitrilotriacetic acid /$C_6H_9NO_6$ (1.5g/L). Its final pH value was adjusted to 7.0 with KOH. The trace element solution was flushed with N_2, and then sterilized using 0.2μm syringe filter.

② Vitamin Solution

The formula of the vitamin solution used in this study was also recommended by DSMZ (Germany). It contains: Biotin (2mg/L), Folic acid (2mg/L), Pyridoxine-HCl (10mg/L), Thiamine-HCl$\cdot 2H_2O$ (5mg/L), Riboflavin (5mg/L), Nicotinic acid (5mg/L), D-Ca-pantothenate (5mg/L), Vitamin B_{12} (0.1mg/L), p-Aminobenzoic acid (5mg/L), Lipoic acid (5mg/L).

③ Mineral buffer medium

The mineral buffer medium is based on PBS (Fish Scientific). The composition of PBS includes NaCl (8g/L), KCl (0.2g/L), Na_2HPO_4 (1.42g/L), KH_2PO_4 (0.27g/L). And

Resazurin (1mg/L), $CaCl_2$•$2H_2O$ (0.147g/L), $MgCl_2$•$6H_2O$ (0.102g/L), L-Cysteine (0.5g/L) and NH_4Cl (2g/L) are added in the medium. The medium solution was flushed with N_2, and then autoclaved. The prepared medium solution was kept in fridge before use.

④ COD-reducing bacteria isolation agar medium

The agar medium for isolating COD-reducing bacteria was based on Wilkins Chalgren Anaerobic Agar (Fluka, Sigma-Aldrich). Its components include: Casein enzymatic hydrolysate,(10g/L), Peptic digest of animal tissue (10g/L), Yeast extract (5g/L), Dextrose (1g/L), Sodium chloride (5g/L), L-Arginine (1g/L). Sodium pyruvate (1g/L), Hemin (0.005g/L), Menadione (0.0005g/L), Agar (10g/L). In addition, K_2HPO_4 (3g/L), Na_2CO_3 (2g/L), $(NH_4)_2PO_4$ (2g/L), L-Cysteine (0.5g/L), $CaCl_2.2H_2O$ (0.2g/L), $MgCl_2.6H_2O$ (0.2g/L), resazurin (1mg/L) and trace elements (10ml/L) were added into the medium. The media was flushed for 15 minutes with N_2 gas. The pH was adjusted to 7 -7.3 using HCl (0.2 M).

⑤ COD-reducing bacteria enrichment broth

The formula of this broth medium was developed from the medium employed in Toerien and Siebert's study (1967). It contains: Peptone (10g/L), Yeast Extract (5g/L), K_2HPO_4 (3g/L), NaCl (2g/L), Na_2CO_3 (2g/L), $(NH_4)_2PO_4$ (2g/L), L-Cysteine (0.5g/L), $CaCl_2.2H_2O$ (0.2g/L), $MgCl_2.6H_2O$ (0.2g/L), resazurin (1mg/L) and trace elements (10ml/L). The media is flushed for 15 minutes with N_2 gas. The pH is adjusted to 7 -7.3 using HCl (0.2M).

⑥ Methanogen selective agar medium

The agar medium developed for isolate methanogens was based on DSMZ 120 Methanogenium Medium (DSMZ Germany). The ingredients of the agar medium include: KCl (0.34g/L), $MgCl_2•6H_2O$ (4g/L), $MgSO_4•7H_2O$ (3.45g/L), NH_4Cl (0.25g/L), $CaCl_2•H_2O$ (0.14g/L), K_2HPO_4 (0.14g/L), NaCl (18g/L), Trace element solution (10ml/L), Vitamin solution (10ml/L), $Fe(NH_4)_2(SO_4)_2•7H_2O$ (2mg/L), $NaHCO_3$ (5g/L), Na-acetate (1g/L), Yeast extract (2g/L), Trypticase (2g/L), Resazurin (1mg/L), Cysteine-HCl•H_2O (0.5g/L), $Na_2S•9H_2O$ (0.5g/L), Mercaptoethanesulfonix acid (0.5g/L), Agar (10g/L). The media was flushed for 15 minutes with N_2 gas. The pH was adjusted to 7-7.3 using HCl (0.2 M).

⑦ Methanogen selective/enrichment broth

The formula of the methanogen selective/enrichment broth was also adapted from DSMZ 120 Methanogenium Medium (DSMZ Germany). Its ingredients include: KCl (0.34g/L), $MgCl_2•6H_2O$ (4g/L), $MgSO_4•7H_2O$ (3.45g/L), NH_4Cl (0.25g/L), $CaCl_2•H_2O$ (0.14g/L), K_2HPO_4 (0.14g/L), NaCl (18g/L), Trace element solution (10ml), Vitamin solution (10ml), $Fe(NH_4)_2(SO_4)_2•7H_2O$ (2mg/L), $NaHCO_3$ (5g/L), Na-acetate (1g/L), Yeast extract (2g/L), Trypticase (2g/L), Resazurin (1mg/L), Cysteine-HCl•H_2O (0.5g/L), $Na_2S•9H_2O$ (0.5g/L), Mercaptoethanesulfonix acid (0.5g/L). The media was flushed for 15 min with N_2 gas. The pH was adjusted to 7-7.3 using HCl (0.2M).

Appendix 7: Preparation Procedure of Bacteria Freeze-dried Stock

The same procedure (Sharma, 2007, pp.201-204) was employed in the preparation of the

freeze-dried stock of COD-reducing bacteria strains and methanogen strains.

① Freeze-dried Stock of the COD-reducing Bacteria

The procedure was employed in the freeze-dried stock of pure COD reducers and the mixed culture of the selected COD reducers. The details of the procedure are given as follows:

The bacteria strain was incubated in sealed serum bottles with the COD reducer broth medium (See Appendix 6 for details). The culture wwas incubated at 35℃ for one week. The incubated cultures were centrifuged at 3,000rpm at 4℃ for 20 minutes, then the cell pellets were collected and re-suspended in sterile mist desiccant (1:3v/v solution of LB broth with 10g/L glucose: horse serum; Sigma-Aldrich). 2ml of each culture suspension was dispensed into freeze-drying vials (Wheaton, vial lyophilisation glass clear 5ml; Fisher Scientific) and sealed with rubber seals, frozen at -80℃ for 24 hours, and freeze-dried for 24 hours in an LTE cryopreservation unit (mini-Lyotrap, LTE) at -55℃. Then the freeze-dried stocks were kept in a fridge at -80 ℃ for further experiments.

② Freeze-dried Stock of the Methanogens

The procedure was employed in the freeze-dried stock of individual methanogen strain and the mixed culture of the two standard methanogen strains (DSM 7222 & DSM 4556). The details of the procedure are given as follows:

The samples of methanogen strains were incubated using the methanogen enrichment broth medium (See Appendix 6 for details) in sealed bottles at 35℃ for three weeks.

Then, the cultures of the methanogen strains were mixed equivalently (v/v). The mixed methanogen culture was centrifuged at 12,000rpm at 4℃ for 5 minutes, then the cell pellet was re-suspended in sterile mist desiccant (1:3v/v solution of methanogen enrichment broth with 10g/L glucose: horse serum; Sigma-Aldrich). 2ml of the suspension was dispensed into freeze-drying vials and sealed with rubber seals, frozen at -80℃ for 24 hours, and freeze-dried for 24 hours in an LTE cryopreservation unit (mini-Lyotrap, LTE) at -55℃. Then the freeze-dried stocks were kept in a fridge at -80℃ for further experiments.

Appendix 8: Preparation Procedure of Sludge Samples for SEM Imaging.

The preparation procedure includes two steps: Fixation (dehydration and drying) and Coating (Dykstra, M. J. & Reuss, L. E., 2003; Kashi, A. M., et al., 2014). The sludge samples were coated with carbon using SEM automatic sputter coater (Agar Scientific, UK). The details of the fixation procedure are given as follows:

a) Fix samples in 3% glutaraldehyde solution for 24 hours at 4℃.
b) Wash with fresh PBS solution and repeat three times.
c) Dehydrate samples by passing them through a series of increasing ethanol concentrations (30%, 50%, 70%, 80%, 90% and 100%).
d) Dry samples in an LTE cryopreservation unit (mini-Lyotrap, LTE) at -40℃.
e) Samples are ready for coating and SEM observation.

Appendix 9: 16s rRNA gene sequencing results for variable regions V1 and V2 of the 29 Isolated COD Reducers

No.	Strain Code	Name
1	C7	*Clostridium sulfidigenes*
NNNNNNNNNCCTGGCTCNNNNNGAACGCTGGCGGCGTGCCTAACTNNNNNNNGTCG AGCGATGAAGCCCNNNNNNGTGAATTAGCGGGGGNNNNNAACACGTGGGTAACCTG CCTTATAGAGGGGGATAGCCTTCCGAAAGGAAGATTAATACCGCATGAAATGTTATT ATCGCATNNNNNNNACATCAATTTTTTAATCCGCTATAAGATGGACCCGCNNNNNNT TAGCTAGTTGGTGAGGTAACCCGG		
2	C10	*Enterobacter aerogenes*
NNNNNNNNNNNNNNNTGNGANNTTGATCCTGGCTCAGATTGAACGATGGCGGCACG CCTAACACATGCATATCGAGCGGTAACACGGAGAGCTTGCCCTCGGTGACGAGCGGC AGACGGGTGAGTCATGCTTGGGACACTGCCTGATGGAGGGGCATAACTACTGGAAA CGGTAGCTAATACCGCATAACGTCGCAAGCACAAAATGGGCGACCTTCGGGCCTCAT GCCATCAGATGTGCCCAGGTGGGTTTAGCTAGTAGGTCGGGTAATGGCTCACCTAGG CGACGATCCCTAGCTG		
3	C13	*Rhodocyclus sp.*
NNNNNNNNCNTNNCTCNNNTTGNACGCTGCGGGCATGCGTTACACAAGCAACTCGA TGGGTTACGCCNCCTTCGTACGCATCCGGACTGGCGAACGGGTGAGTATTGCATCCG ACAATGCCCTGAAGTCCGGGATAACTTAGCGACAGTAAGGCTAATACCGCATCGATC GTGCGCAGGAAAGCAGTGGACCACCGGGCCTTGCCCCTTGGGAGTGGCCGATGTCTT AACAGCTAGTTGGTGAGGTACTTCTAACGACGGCAACGATTTGTAGCGCGTCTGCGA GAG		
4	C15	*Proteus vulgaris*
NNNNNNNNNACNNNNNCGNCAGNCCTAAGACATGCAATCCAGCGGTTACAGTAGAA AGCTTGCATTCTTGCTGACGAGCCGCGGACGGGTGAGAAATGTATGGGGGTCTGCCC GATTGAGGGGGATAACTACTGGAAACGCTGGCTAATACCGCATGACCTCTACGGACC AAAGCAGTGGCTCATCGGACCTAGCGCTATGGGATGAACCAATATGGGATTACCTAG TAGGTGAGGTAATGGCACACCTAGGCAACGATGTCTAGCTTTTCTGAGACAATGATC AGCTACACTAGC		
5	C18	*Escherichia coli*
NNNNNNNAGNNNNNGTNNNNANNGCNNCATAGACGCTGGCTACAGCCCGGCAACA AGGAACTCTACCGTGAACTCGTAGAGACTAGCTTCTCGCCGACAGGTGGGGGAGGG GTCAGTTTTTTCTGGGAACCTGGCTGGTGGAGGCCGATAACTGCTGGAATCGGATGC TAGTACCACATAGACTCGCAAGTACAATGAGCGAGACCTTGGCGCCTCTTGAGATCT GATGAGCCCAGATGCCATTATCTATAAGGGGGGGTAACCGCTCACGTAGGGGACC		
6	C19	*Pseudomonas sp.*

NNNNNTNNNNCTNGNNNGACANNCACCTATNNNNANNNNNNTCACATGCTGGTCGA GCATTTGAAACGAGATGGCCCCTGCATCCAGCGGCGGACTTGTGTGTAATGCCTGGG AATCTGCATGGTAGTGGCGGATAACGTACGGAAACGCTCGCTAATACGGCATAGGTC CTGAGCTAGCATGTGAAGGATTTTCGGACTTCCCGCTATCAGGTGACGATGGGTCGG TTTAGCTAGTAGGAGTGGTAAACGCCTCCCAAGGCGGCGATCCGTTACTGTTCTGAG		
7	C20	*Pasteurella sp.*
NNNNNNNNNNNGCGCCNGTTTCAGAANNNNNGACANNCTTCCGCCATGCAACCCG AACGCTAACATATAGAAGCTTGCTTCTCTGATGACGAGTGATAGACGAGTGAGTATT GCATGAGAATCTAGCTCATGGAGAAGGATAACTACGCGACACTGTAGCTTATACTGC GTAGGCTGGAGAGATGAAAGTGTGAGACCAATTTATCCTGGGACGTTACGATGAGCC CAAGTTTAGGCAGGTAGTTGGTGAGGTATTGCCTCACCATGCGGACGATCTCTAGCA		
8	C21	*Citrobacter freundii*
NNNNNNNNNNCCANNNCAGACTGAACTCTGATGGCAAGCCCAATACATGCAGGTCG ATTGCTAGCACAAAACAGCTTCGTCTATGGATGACGAGTGCTGGACGATTGAGTACG GTCTAGCTTTCTGCCCGATGCAGGGGGATTACTACTCGAGGCGGTAGCTCCTACCGC ATATCGTCCCAAGACCATTGAGGCCGACCTTCGGGCCTCTTGCCATCTGATGTGCCCA GATGCGATTAGCTAGTGGGTGCCGTAACGGCTCACCTATGCGACGCTCCATA		
9	C22	*Enterococcus.sp.*
NNNNNNNNATNNNNNGCANNCANAATACCATCAAGTTACACGCTAACTCTTACACC GCAGCTTGCACGATCGATAGAATTCCTTTGGCGAACGGCTGAGTCACACATGTGTAA GCTGGCCCTCAAAAGTTGATACAACTTGTAACCAGCTGCTAATACCGTATAACACTA TTATCCGCATGGAAGACAGTTGAAAGGCGCTTATGCGTCACTGATGGTTGGACCCGC GGTGCATTCTCTAGTTGGTGAGGTGCCGGCTCAATAAGCCCGCGC		
10	C28	*Bacillus sp.*
NNNNNNNGGANNNNCGNTGCGNNNNNNCCCAAAACATGCATGTCGAGCGGCCTAAT GGGAGCTTACTCCCGTTTGTTAGCGGCACCCTGGAGCGTAACACATGGGCAACCTTC CTGTAAAGCTGGGATCACTTCGAGAAAGCGTTGCTAATACCGCATGACATATTGGAA CTCCTGTTCCGCTATTAAAAGATTGCTTCGGCTATCACTCACAGTTGAGCGCGCGGCC CTTTAGGTAGTTTGTGAGGTAACGGCTCACCAGGGCGATGATGCCTATCCGACCTGA GGGGGTAATCGGATT		
11	C37	*Trabulsiella guamensis*
NNNNNNNCGNANNNTTANNCATNCNNGTCGAGCGGCAGCGGGGNNAAGCTGCTTTC GCGCCNNNGAGATGCGGACGGCGTGGTAATGTCTGGGATACTGCCTGATGCAGGAG GATATCTAGTCGACACGGTGGCTNATACCGCATATCGTCTTCGCCCCAATGTGGTTG ACCTTCGAGCCTCATGCCATCGAATGTGCCCCGATGGTGTTAGCTAGTAGGTGANGT AACGGCTCAGGTAGGCGACAATCCNTAGCTGTTCTGAGCGTATGGCCTA		
12	C38	*Clostridium sporogenes*
NNNNNNNNNNCNNNNNCAGGACGTACGCTCGCGGCGTGCGCAACACATGCAAGTCT AGCGATGAAACTTCCTTCAAGAATTGGATTAGCTGCGGACGGCTGAGTAACACGGGG TTAACCTGCCTCAGAGCCTTCCGAAAGGAAAATTAAGACCGCATACCATAAGCGAAT CGCATGTTNTTCTTATCGAAGATTTCTTGCTTTGAGATGGACCCGCGGCGCATTAGCT NGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGNAGCCGACCTGAGA		

13	C41	*Rhodocyclus sp.*

NNNNNNNNNTNNAGCNNNNNANGCTTTCNATAGTCGAACGGTAACGCGNGGGACC
CCNTGGCGACGAGTGGCGAAGTTTCCGGACTGGCATCGGAGCGTGCCCTGAAGTGCG
GGATAACGTAGTGAAGTTTACGCTACGCCCGCATATTCTGTGAGCAGGTTAGCAGGG
GATCTTAGGACACTGCGCTTTGGGAGCGTTCGATGTCGGATTTAGTAGTTGGTGAGC
TAACAGCTCACCAGGGCGACGATCCGTAAAGGGTCTGAGACGACGATCCGCC

14	C43	*Clostridium sporogenes*

NNNNNNNNNGAGTGGATTAGCGGCGGTCAGGTGGGGGATATTAACACATGCCCCGT
CGAGCGGTGAAGCTTATACCGCATAGTGGATTAGCGGCGGACGGGTTTCTTATCAAA
GATTTATTTGCCTCAAAGTGGGGGATAGCGCATTAGCTAGTTGGTAAGTACTGAGAC
CCCAAGGCAATCGCACGTAGTTCTTATCAAAGGGTGATCGGTTTGAGATGAACTGAG
ACACGGTCCAGACGTTGGTAAGGCGCAATGGGGGAAACCCTGACGACCTGAGA

15	C45	*Anaerobacillus*

NNNNNNNNNNACGANAACTGGCGCCGTGCCTGATAACTGCAAGGGGAGCGGAATAG
ATTGGAGCTTGGTTCCTAACACTTACCGGCGAACGGGTGAGTAACACTTGAGCAACC
TGTCTGTAGACTCGGATAACTCGGCCATACCGAAGCTAATACCGGATATTCTATGGA
GCGTCATGGTTCTATAGTAGAAGATGGGCTTAACCTAACTCTACACCTTGGGCCCAG
GGCGCATTAGCGAGTTCGTAAGTGAAGGGCTTACCAAGGCGACGA

16	C46	*Desulfovibrio baculatus.*

NNTNGGNNNNCNANNNNNNNANTNCNNTCACAGCTGCCGTCGTGCCAATCACATGC
TATTGCTACTAGTAAGCGCGTTAGCGCCTGCGTATGCGTGGAGCTCGTGTGAGTAAC
GCGTGCGTAATCTACCCTGTGATTCGGCGTAACTCTACCAAACGTGGACGATATACG
GGATAGTTTGACTTTAATTAAGAAGTCGCTAAAGGATGCATCTGCATATGGACTCGT
CCGTGAATGAGCCCGCGTATCATTAGCTAGTTGCTGGGGTAATGCCGTACGAAGCCA

17	C47	*Pseudomonas sp.*

NNNNNTNNNNNNNNCNNNNNCGCTGACGGCCGGCCTAACACATGTAAGTCAAGCGG
ATGAGTGGGGCTTGCTCCCTGATTCAGCTGCGGAAGGGTGAGTACTGCCTAGGAGTC
TGCCTGCTAGTGGTTGACAACGTTTTGCAAGGAACGCGAATACCGCAAACGTCCTAC
GGCAGAAAGTGGGGGGTCTTCGGACCTTACGCTATCAGATTAGCCTCGGTCGGGTTA
GCTAGCTGGTAAGGTAATTGCTCGCCAACGCGACGATCTGTAACTGATCTGAGAGCA

18	C50	*Clostridium sporogenes*

NNNNNNNNNNNNNNNNTCAGCACGATCGCTGGCGGAACGCTTAACTGATGCAACGC
GAGCTGTGAACCTACCTTCAAGGGGTGGATTAGCAACGGGAGGGTGAGTCGCACGT
GACTAACCTGGGTCAAAGTCAGGGAAAGCCTTCCGAACGGATGGCTAATACTACATA
ACTTAAGAGGGCGCATTTTTTTCTTATCAATGATTATTTGCTTTGAGAACGACGAGCG
GCGCATTCTCTATATGGTAAGGTTGCGAGTTACCAAGCGAACCGTGCACAGCTAACC

19	C51	*Escherichia coli*

NNNNNNNNNANNNNNANNGTATTGGTGCCTTCGGGAACTGTGAGACAGGTACACAT
GCAATGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTTATCTGTCTGGGAAAGCGGTCCGGGGAGAGGGATAACTACTGGAGCCAGTGATA
AACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTA
CACACGTGCTACAATGGCGCATACGTAGGTGGGGTAACGGCTCAGAGCAAGCGGGA

20	C53	*Bacillus firmus*

NNNNNNNNNNNCAGGNNNAACNCTGGCAACGTGCCTATTACAGGCACGTCGATCG
GACGGGATGGGAGTATGCTCGCAGACGGTCAGCAATGGACCAGTGAGTAAGACGTGT
TCAACCTGTCTGTAAGACTGCGATAATTCCAGGAAACCGCGGCTAAGACCGCATAATT
CTTATCCTCGGATGAGGNTAAGNTGAAATATGGCATCTGGCTATCACCTACAGATATGC
CCCGGGCGCANTAGCTAGATGGTGCGGTGACGGCTC

21	C54	*Clostridium pasteurianum*

NNNNNNCNNNNNNANTNNNCTAGCGGCGGAGGCGTGCTTAACACATGTTAACCTGCC
TCGAAACCTTCGATAGCCTCCCGATTTCTAGCTTAAGACGGGTGAGTATTACAGGCTTC
GAATTGCCTCAAAATTAAAGGAGGAATCCACTTTGAGATGGTAATACCGCATTTGAGC
GGACCCGTGGCGCATTAGCTACTAAGGGAGTAAACCGCTATCAAAGGCACGACGTGG
CGCACCAGCTCGTTGGAGTGATAACGCCTCACCAAGCTGAGGAGGTC

22	C55	*Clostridium perfringens*

NNTNNNTTNNNNNGTAAAGGGAGGCTCAGGATGAAATTAAGTGGCGTGCTTAACACA
TGGGCTCAACTTGGGTGAGTTTCTTCGAACTGGTGATTAGCGGCGGACGGGTGAAGA
GTGGAAGGGTAACCTGCCTCATAGAATGCAATAGCATTAGGAAGAAAAGATTAATACC
GCATAATGATCTGGACTGGCATCATCATTCAACCCCAGTAGCGTGAGGAGGAACCCGG
ATCAGATGCCTCATAAGCTAGTTCCTGGGGTAACGCCCTCATGGTGTGGGCGT

23	C56	*Micrococcus flavus*

NNANNNANGGNGCNNNNNNANATCTTACACATGCAAGTCGAACGATGTATACGTGAG
TAAGCTGGGCTTGATTAGTGGCGAAGCCCTGAGTAACACGTCTGTAACCGGCCCTTGA
CTCTGTGATAAGCGCGGGTAACTGAATCTACTATCGGATAGGGGCGCCTGTCGGGCGA
CGGGGCTTGGTAAGATGTCTCGGTCTTAGATGGACTCCCGGGGGATCAGCTCCTCGGA
GAGGAAACGACTCCCCAGGCCGACGACGGCTAGCCAGACTCAGAGGGGGGCCGGCC

24	C57	*Escherichia coli*

NNNNNNNNTTNNNNNNGNNANTTGAACGCTGTCCCCGGGCCCTAACACATGCTGCAT
GGCTGTCGTCAGCAACTGATGACCTCTTTGCTGACGAGTGGCGGCAACGAGCGCAAT
GTCTGTCAAACTGGGATATGGAGCCGGCCAACTACTGAAAGGAGACTTAATACCGCAT
AACGTCGCAAGGTGAAAATGACGTCAAGTCGTCATGGTTCTTACGACGATGGCTACA
GATGTGCTACAGTAGTAGGTGGTGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCA

25	C58	*Pseudomonas sp.*

NNNNNNNNNTCTTGNCTCAGCTTGAACTTTGGGGGACTGCCTATTACATGCAAGTCCA
GCGAATGAGTGGAGCTTGCTCGGTGATCGAGCGTCGGACGGATGAGTAATGTCCTGGA
TCTGCCTATTAGTGGGGGATATCGTTCGGACAGGAACTCTAATACCACATACGTAAGAA
AGGGGGGGACTTCCGTTCCTTGCGCGCTCAGATGAGCCTAAATCAGATTAGCTAGTTG
AAGAGGTAATGTCTCACCACGGCCTCGTTCGGTAACTGATCTGCGAGAATGAAGCATC

26	C59	*Bacillus sp.*

NNNNNNNNNTNANNNCGCCATATACGTGCCACTCGAGCGGATGGATGCGAGGTTGCT
CAAGGATGTTACCTGCGGACGGGAGAGTCCCACGTGAGTAAGCTGCTTGTAACACTG
GGATAACCCCGGGAAACCGGGGCTTTAACCGGATGCTTTTTTAAAGCTCATCGTTGGA
ACCTAAACAGTTGCTTCGGCTACCGCTCTCAGATGCACCCGCTGCGCAATAGCTATTTA
ATGAGGTCGTCGCTCACCCGGGCGACGATGTGTAGCCGAGGTGAGAGGGAGATCCCC

27	C60	*Clostridium sulfidigenes*

NNNNNNNNNGTNNNATCGAGTNGAACGCTGGAGCCGTTCGGCGTGGATGAACGCG
GGACGGGCGAGTACCAGGTGACGAACTTGCCTTATAGAGCCGGATAGTTTTCCGAGA
GGCAGATAAATATTGCAGGAAATATCTTTAGCTCACGGTAGAAACATCAAAGTAGCAA
GCCGTAATAAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCCCAACAA
GGCGACGATACTGAGCCGACTTGAGCCCATGATCGGCCACATGGAAACTGAG

28	C62	*Bacillus sp.*

NNNNNNCGNNNNNNNNNNNCCAAGTCGAGCTAATCTTTGGGACCTTGCTCCCTTTGG
TTAGCGGCCGACGACGTGGGCAACCTGCGTGTAAGACTTTGATAAACTCGGGAAACC
GGAGCTAATTACGGATAATTCATTTCCTCTCATGAGGAAATGCTGACAGACGGTTTAGG
CTGTCACTTACAGATGGCCCCGCGGCGCATTATCTAGCTGGTGGGGTCACGGCTCAAG
AATGCGACCCTGCGTAGCATACCTGAGAACGTGATTGGGTACACTGAGC

29	C65	*Escherichia coli*

NNNNCNNNNNCGNNAAAAANNNNNNNNCTNNNCNGNAGAAANCNACCATTCAAGT
CCATCGGTAGAAGGAAGTTGATCGCTCTTTGTTGACGAGTAGCGCACGGTAGAGTACT
GTCGGGAAAACGGCATGATGGCGGTGGATAACCAGCGCAAACGGTGACTACTACCGT
TTAACGTCGCGCGACCAAAGAGCCGGACCATCGGGTCTCTTACTGTCCGATGTGCCCA
GATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGAT