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The toxicity of *o*-cresol and 2,4-dimethylphenol on anaerobic microbial associations

By

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Abstract

The study of anaerobic bacterial growth associations plays an important role in the analysis of the toxicity and effect of pollutants. These associations can be found in the environment, for example in groundwater or wastewater treatment plants or even in the gut of animals.

The aims of this study were to compare the toxicity exhibited on anaerobic growth associations by two similar alkylphenolic compounds, *o*-cresol and 2,4-dimethylphenol (DMP), individually and combined, and examine the specific effect these compounds had on individual bacterial groups within the consortium.

Through the use of anaerobic batch cultures, the inhibitory concentrations and effects of *o*-cresol and 2,4-dimethylphenol on anaerobic processes were analysed. Concentrations of *o*-cresol ≥ 1.85mM were shown to have an inhibitory effect on sulphate reduction, acetogenesis and methanogenesis. At concentrations ≥ 7.4mM complete inhibition of sulphate reduction was noted and the acetogenic and methanogenic processes were greatly reduced, concentrations ≥ 9.3mM appear to cause complete inhibition of methane production. 2,4-dimethylphenol inhibited bacterial groups at concentrations ≥ 0.41mM; complete inhibition of sulphate production was observed at concentrations of DMP ≥3.28mM. Although methane production was significantly reduced at the highest concentration (4.1mM) complete inhibition was not observed. It was found that DMP was more toxic to all the bacteria and archaea within the consortium than o-cresol.

Use of continuous flow systems allowed for the separation of the microbial groups within the consortium allowing for the analysis of the direct effect of o-cresol and 2,4- Dimethylphenol. Analysis of the results indicated that the compounds inhibited all the microbial groups, although with varying

degrees of success. It was observed that acetoclastic methanogenic bacteria are more resilient to the toxic effect of the compounds than hydrogenotrophic methanogens and sulphate reducing bacteria.

The combined toxicity of these compounds was analysed utilising batch cultures. The combined toxic effect on the growth consortium was observed after addition of both of these compounds to batch cultures. The results showed that, when combined, these compounds exhibited a toxic effect on the consortium, which was synergistic in nature.

This study has highlighted the toxic effects of o-cresol and DMP on anaerobic consortia and the importance of the synotrophic relationships between the different bacterial and archaeal groups. It is important that work in this field is carried on to develop a better understanding of the implications of toxic chemical spills and their impact on the groundwater environment.

Chapter 1

Introduction

1.1 Introduction

Groundwater is found below most of the earth's surface in a zone below the water table known as the saturated zone. Above the water table is the unsaturated or vadose zone, which consists of voids in rock and soil that contain both water and air. The saturated zone contains only water in cracks and crevices of rock and in spaces between soil and rock particles (Bower 1978). Figure 1.1 depicts the groundwater system.

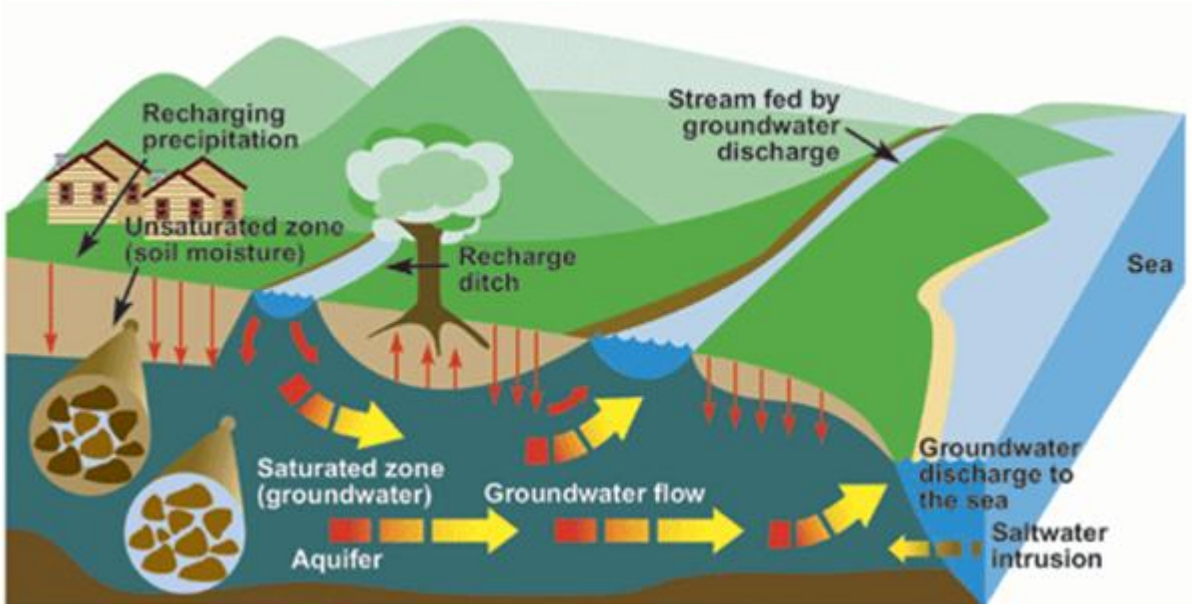


Figure 1.1: The ground water flow, and both the saturated and unsaturated zone (Environment Canada, 2013)

The flow of water varies greatly with the type of material that it has to travel through in the saturated zone. Normally the water flows very slowly, although its speed can increase if the rock formation is very permeable. In the saturated zone ground water can be found in an underground formation of permeable rock or loose material known as an aquifer (Todd 1980). Aquifers vary in size, from only a few acres to thousands of square miles and can be considered as underground reservoirs, which can be tapped by a well to produce useful quantities of freshwater. This freshwater is commonly

used as a source of drinking water, (Kampfer *et al.,* 1991) and, indeed, the majority of drinking water worldwide is extracted from groundwater. In China groundwater is used as the primary source of farmland irrigation (Zhang *et al.,* 2008)

Since groundwater can be used as a source of drinking water, it is important that chemicals, which could be potentially hazardous to human health, do not contaminate it. It is also important to note that groundwater is part of the hydrologic cycle and therefore it eventually makes its way into streams and rivers which lead into the sea, from which it evaporates and forms clouds. Thereafter, when it rains some of the rain will seep through the ground and enter the saturated zone, a process known as recharge. This means that should groundwater become polluted with toxic chemicals, it is not only the drinking water that is affected, aquatic life in streams, rivers and the sea and land life (plants and animals) may be affected from contamination from the flow of the water or rainfall. Wycisk *et al*., (2003) stated that the pollutants found in groundwater could spread to uncontaminated areas and endanger receptors like drinking water wells.

1.2 Groundwater contamination

The contamination of groundwater can occur in both agricultural and industrial areas. It is of major concern and can have wide spread ramifications for human and animal life. In some areas especially developing countries poor legislation and monitoring allow for uncontrolled dumping of industrial and domestic effluent, which can enter the water table (Rahman and Lee 1997; Reyes-Lopez *et al*., 2008). Emerging trace organic contaminants from pharmaceutical waste chemicals are found in the groundwater environment. These chemicals enter the groundwater environment from

wastewater treatment plants that are not functioning correctly and also by direct dumping of the chemicals in to the water eco system (Tran *et al*., 2013).

Groundwater contamination can occur from a number of sources, both point and non-point sources. Some of the major sources of pollutants are shown below (Todd, 1980):

1.2.1 Point sources

1. Septic tank systems
2. Municipal landfill sites
3. Sludge disposal areas at petroleum refineries

1.2.2 Non-point sources

1. Fertilisers on agricultural land
2. Pesticides on agricultural land and forests

Both of the above pollutants enter the environment due to being carried through the soil by rainwater.

Of these sources improper storage, underground tank leakage and spillages are the most common routes for environmental contamination of ground water (Hudak *et al.,* 1995; Nadim et al., 2000).

1.3 Groundwater Microbes

Groundwater contains both anaerobic and aerobic microbes (Colarieti *et al*., 2002) that contribute to the breakdown of contaminants that enter the

groundwater environment. For example, if landfill leachate finds its way into groundwater, by leaching from unlined landfill sites (Johnston *et al*., 1996), heterotrophic synotrophic bacteria may degrade the organic chemical constituents (Smolenski and Sulfita, 1987). These processes help to keep the water clean and free from any possible contaminants that could make their way into the drinking water system or the environment as a whole.

The microbial food chain found in groundwater is heterotrophic. Organic matter from the sub-surface environment is the source of carbon for the bacterial metabolism. As the depth of the ground water increases the dissolved organic carbon decreases, this in turn limits heterotrophic groundwater metabolism. Alfriedera *et al.,* (2009). It is possible that microbes in deep sub surface environment utilise H_2 (electron donor) and CO_2 (electron acceptor) from water rock interactions, these microbes are likely to be archaea.

Harmful chemicals that enter the ground water environment may be toxic and exhibit an inhibitory effect on the bacteria present in the water. For example, the compounds *o*-cresol and phenol produce a toxic and inhibitory challenge to the bacteria (Holmes *et al.,* (2002)). They are common constituents of polluted groundwater and are found in many chemicals including crude oil and pharmaceutical by products. (Spence *et al*., 2001; Sauter and Licha, 2002; Asan and Isildak, 2003).

The bacteria found in groundwater are mostly anaerobic (although aerobic and facultative bacteria are also present) as there is not a plentiful supply of oxygen (Hansen *et al.,* 2001). Any dissolved oxygen present in the water is

found near the surface and is rapidly depleted by aerobic bacteria, which creates an anoxic environment (Parkes and Senior, 1988). Therefore the most important bacteria present in groundwater are anaerobic as these are the bacteria that have to deal with any pollutants present. This makes the degradation of chemicals a slow process. It is known that under aerobic conditions a single microbial species may degrade a chemical, whereas under anaerobic conditions a range of different microbial types work in conjunction to completely degrade organic compounds (Smolenski and Sulfita, 1987; Parkes and Senior, 1988). According to McInerney *et al.,* (2009) anaerobic metabolism differs from aerobic metabolism due to it being completed by a consortium of synotrophic microbial species as opposed to aerobic metabolism where only a single microbial species is required to mineralise organic compounds.

A number of different types of bacteria have been shown to be present in ground water. The main groups are

1. Hydrolytic and fermentative or acid forming bacteria
    - Hydrolytic and fermentative bacteria utilise enzymes that enable the breakdown of large polymers into their respective monomers e.g. lipase for lipids. The breakdown of these larger molecules produces long short chain fatty acids, propionate and butyrate are most abundant. Acetate is also produced in this stage although in a smaller scale. (McInerney *et al*., 2009)
    - These bacteria use sulphate, nitrate, manganese and iron as electron acceptors. (Dyer 2003; Thornton et al., 2001).

2. Hydrogen producing acetogenic bacteria (McInerney *et al*., 2009; Kotelnikova (2002); Hansen *et al*., (2001))
    - generate acetate by the oxidising the longer chained fatty acids to acetate hydrogen and carbon dioxide.

3. Methanogens (Kotelnikova 2002; Hansen *et al*., 2001; Christensen *et al*., 2000)
    - Acetoclastic- generate methane and carbon dioxide from acetate. ($CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$)
    - Hydrogenotrophic- utilise hydrogen and carbon dioxide to generate methane. ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$)

These bacteria work in conjunction with each other, to completely mineralise pollutants to carbon dioxide, methane and water. For example: if polymeric substances such as proteins, polysaccharides and lipids enter an anaerobic environment they are first hydrolysed to yield their respective monomers, amino acids, sugars and long chain carboxylic acids. These products are in turn fermented to produce acetate, carbon dioxide, hydrogen, short chain carboxylic acids and alcohols. The acetate, carbon dioxide and hydrogen are used directly by the methanogens. The remainder of the fermentation products are degraded further by acetogens. The end result is that the compounds are fully metabolised to produce water, methane and carbon dioxide (Watson-Craik *et al*., 1991).

The first stage is hydrolysis, fermentative bacteria secrete hydrolytic enzymes which hydrolyse complex insoluble organic polymers e.g. cellulose, into soluble monomers in the form of sugars and alcohols. The second stage involves the acetogenic bacteria converting the products of the first stage into simple organic acids, carbon dioxide and hydrogen. According to Molino *et al*., (2013) the most abundant acids produced are acetic, propionic and butyric. Further degradation of the propionic and butyric acids occurs from sulphate reducing bacteria and acetogens to produce acetic acid. The acetic acid is then converted to methane by the acetoclastic methanogens. Further methane is produced by the hydrogenotrophic methanogens, which utilise the bi-products of the previous stages hydrogen and carbon dioxide, (Molino *et al.*, 2013).

The bacterial groups present in groundwater have synotrophic relationships with each other forming a bacterial community that relies on the work of each group.

Synotrophy is essential for the complete degradation of polymeric substances to carbon dioxide and methane (McInerney *et al*., 2009). According to McInerney *et al*., (2009), anaerobic synotrophy is a thermodynamic interdependent lifestyle with the breakdown of a compound occurring when the end products of the degradation (hydrogen, methane and acetate) are kept at a low concentration. Therefore if the end products of the earlier stages of anaerobic degradation are not utilised the whole process will fail.

When larger molecules e.g. proteins and cellulose are hydrolysed and fermented in to smaller products, hydrogen is produced as a bi-product, if too much hydrogen is allowed to accumulate the hydrogen partial pressure becomes too great for the community to function and causes the anaerobic degradation to cease or slow. Due to the synotrophic relationship the hydrogen-scavenging bacteria within the communities utilise the hydrogen, these bacteria are the sulphate reducers and the methanogens, although they can only utilise the hydrogen if there are available carbon sources. The carbon sources they need, are produced by the fermentative and acidogenic bacteria (Mizuno *et al*., 1998). In the case of the sulphate reducing bacteria, hydrogen, organic matter and sulphate are utilised as electron donors and electron acceptors respectively to give sulphate reduction (Mizuno *et al*., 1998). A bi-product of sulphate reduction is the formation of hydrogen sulphide, which is known to inhibit methanogenisis at high concentrations.

The synotrophic nature of the bacterial communities allows them different routes for the degradation of organic carbon. For example, ammonia produced from the biodegradation of proteins can inhibit acetoclastic methanogens. This in turn causes a build-up of acetate and hydrogen which in turn can affect other microbial process, but the acetate is converted to hydrogen and carbon dioxide by synotrophic acetate oxidising bacteria, these bacteria are thermodynamically driven by the hydrogenotrophic methanogens which produce hydrogen by utilising hydrogen (Tran *et al.*, 2013).

Anaerobic bacteria utilise alternative electron acceptors other than oxygen. They are known to use a whole range of different electron acceptors, for example: manganese, iron, nitrate, sulphate and carbon dioxide or fermentative material, (Colarieti *et al.*, 2002). Table 1.1 shows the redox reactions found in the anaerobic groundwater environment.

Table 1.1 Electron accepting redox reactions

Electron accepting processes	Intermediate and final products
$CO_2 + 8H^+ + 8e^- > CH_4 - + 2H_20$	CH_4
$SO_4^{2-} + 8H^+ + 8e^- > S^{2-} + 4H_2O$	H_2S, HS^-, S^{2-}, S^o
$Fe^{3+} + e^- > Fe^{2+}$	Fe^{2+}, Fe^{2+} complexes
$Mn^{4+} + 2e^- > Mn^{2+}$	Mn^{2+}, Mn^{2+} complexes
$NO^{3-} + 6H^+ + 5e^- > 1/2N_2 + 3H_20$	NO^{2-}, N_20,N_2

Adapted from Ludvigsen *et al.,* (1998)

In the groundwater environment the anaerobes exists in small pockets or micro-niches, which depend on the presence and quantity of their electron acceptors and nutrients (Thornton, 2001). Since the anaerobes can utilise

many organic pollutants as carbon sources, then microniches can be found near pollutants plumes. These plumes have active redox potentials, this is due to the organic carbon being oxidised by microbial process and in turn the terminal electron acceptors present being reduced. For example sulphate-reducing bacteria utilise the carbon source and reduce sulphate to hydrogen sulphide. Christensen *et al*., (2000) states that most redox reactions found in contaminated plumes are due to microbial activity and involve the conversion of organic matter. Since the microbial communities are diverse different redox potentials are observed throughout the plume. The length of time a plume persists or has microbial activity is due to a number of factors, such as the extent of the pollution, the groundwater flow rate and finally the geochemistry of the aquifer (Christensen *et al*., 2000).

Redox plumes play an important role in identifying groundwater contamination, the redox conditions of groundwater contaminated with organic carbon compounds will become strongly reduced as microbial reactions utilise the organic carbon substrates as proton donors, and reduce the oxidised electron acceptor species that are present in the groundwater environment. Groundwater pollution plumes contain a number of different redox conditions/environments, which are distributed physically throughout the plume. The redox conditions closest to the source of pollution are reduced, as the distance increases from the source, the conditions become more oxidised and eventually the redox conditions are similar to a pristine aquifer (Christensen *et al*., 2000).

According to Naudet *et al., (*2004) the analysis of the redox conditions of groundwater is important for the characterisation of the contamination

plumes and for the development of remediation technologies. When strongly reduced landfill leachate enters an uncontaminated pristine groundwater environment it causes changes in the redox potentials of that environment. The reduced leachate contains organic matter and electron acceptors; these are catalysed by the groundwater bacteria causing redox gradients to be formed. The greater the distance from the contamination, the greater the redox potential, (Naudet *et al.*, 2004).

1.4 Pollutants

The types of hazardous pollutants that find their way into ground water include pesticides, fungicides, polyaromatic hydrocarbons (PAH's), chlorinated aromatic hydrocarbons (CAH), BTEX compounds (benzene, toluene, ethylbenzene and xylene) and phenolic compounds. (Edwards and Grbic-Galic, 1994; Colarieti *et a*l., 2002; Sauter and Licha, 2002; Dyer, 2003).

Pesticides and fungicides enter the groundwater environment due to rainfall infiltration through the soil to the water table. BTEX, PAH, CAH and phenolic compounds are found in crude oil and enter groundwater due to improper storage, spillage and improper disposal. These aromatic chemicals are also used in various industrial processes, and it has been found that leaking underground storage tanks present a huge problem causing environmental contamination (Caldwell *et al.,* 1999; Maliyekkal et al., 2004). Most of these chemicals are considered toxic and are either known or potential carcinogens, making their presence in groundwater of considerable concern.

In this project the effect of two alkylphenol compounds on the anaerobic bacteria present in ground water will be studied. The chemicals that will be studied are the methylated phenols, *o*-cresol and 2,4-dimethylphenol. The structure of which are given in Figures 1.2 and 1.3, respectively.

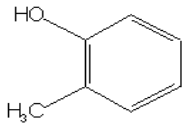


Figure 1.2: *o*-cresol

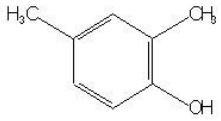


Figure 1.3: 2,4dimethylphenol

These two compounds are of environmental significance since:

1. they can both readily enter the groundwater environment
2. they both have considerably high aqueous solubilities (Table 1.2.)
3. both of the compounds are known to be toxic (Devillers 1988; Sulisti 1994; Holmes *et al*., 2002; Kahru *et al*., 2000)
4. they are known to migrate considerable distances from their entry point into the system (Guanhge *et al*., 2000)
5. little is known about their environmental effect, as previous studies have focused on other compounds such as BTEX, PAH, Chlorophenols and higher chained carbon molecules
6. there has been little research into their eventual fate, e.g. their potential degradation in an anaerobic ground water system

where there are limiting amounts of nutrients and electron acceptors for the bacteria to utilise.

These two short-chained alkylphenols (SCAP) are major constituents of coal liquefaction products and crude oil (Sauter and Licha, 2002). Alkylphenols such as these, with low molecular weights (*o*-cresol, 108.14gmol^{-1}; DMP, 122.16 gmol^{-1}), have a relatively hydrophilic nature, which allows them to readily separate from a petroleum complex and enter formation waters (Taylor *et al*., 1997). According to Sauter and Licha (2002), SCAP compounds can be found in groundwaters which have been affected by spillages close to oil exploitation sites and coal liquefaction plants, due to SCAP compounds having high aqueous solubility and specific oil/water partitioning behaviour. Smolenski and Sulfita (1987) stated that alkylphenols are important examples of groundwater contaminants and are produced from a wide number of sources. Creosote or oil contaminated groundwater contains a mixture of phenolic compounds (Flyvbjerg *et al*., 1993). There are 35 SCAP compounds, which can all be found in the environment.

Phenolic compounds, such as *o*-cresol and 2,4-Dimethylphenol, are also found in pesticides, wood preservatives, dyes and synthetic intermediates (Asan and Isildak, 2003, Chan *et al*., 2005). They are also used in the production of various chemicals such as disinfectants. Therefore, if the compounds containing the phenolics are not used in a responsible manner and disposed of correctly, they may enter the environment and eventually make their way in to the groundwater system. Phenol and its derivatives are one of the most common types of toxic organic contaminants which exhibit hazardous effects on the environment (Ruey-Shin and Shang-Yuan, 2006).

The reason for the high solubility of SCAP compounds is that they have the ability to partake in hydrogen bridging systems (Sauter and Licha, 2002). Hydrogen in water is attracted to the hydroxyl group of the phenol due to the oxygen being an electron rich atom, and this causes the formation of an electrostatic bond between the SCAP and the water. The solubilities of some SCAP compounds are shown in Table 1.2.

Table 1.2: The solubility (g/l) of SCAP compounds at 25°C (Varhanickova *et al*., 1995).

SCAP	Solubility (g/l)	SCAP	Solubility (g/l)
phenol	102.1	3,5-dimethylphenol	6.7
o-cresol	26.8	2-ethylphenol	14
m-cresol	19.6	4-ethylphenol	8
p-cresol	22	2,3,5-trimethylphenol	0.9
2,3-dimethylphenol	6.4	2,4,6-trimethylphenol	1.4
2,4-dimethylphenol	8.2	3,4,5-trimethylphenol	1.5
2,5-dimethylphenol	3.8	4-nPropylphenol	1.3
2,6-dimethylphenol	6.2	2-isoPropylphenol	4.4
3,4-dimethylphenol	7.2	4-isoPropylphenol	3.3

From Table 1.2 it can be seen that the addition of one or more methyl group lowers the solubility of the compounds. Due to the high solubility of SCAP compounds and the fact that they are fairly small molecules, they can travel

great distances mainly unretarded in ground water (Sauter and Licha, 2002), eventually end up in the drinking water system.

Of all the alkylphenols found in the environment, the three-cresol isomers are the most abundant. In a study of petroleum systems by Taylor *et al*. (1997), it was found that the most abundant cresol was *o*-cresol and the most abundant xylenol was 2,4-Dimethylphenol. Other studies have found *o*-cresol to be the most recalcitrant (Boyd *et al.,* 1983; Smolenski and Sulfita, 1987; Flyvbjerg *et al.,* 1993; Spence *et al*., 2001) of the three cresol isomers in anaerobic environments and indeed also when compared to other phenolic compounds. This means that it could be very persistent in the environment and with its fairly low molecular weight it can migrate rapidly. A study by Harrison *et al*., (2001) showed that all three cresol isomers were found in a contaminated groundwater plume next to a coal tar distillation plant near Wolverhampton U.K. Of the three cresol isomers o-cresol (Figure 1.2) was the most abundant with a concentration of 13.4mM, which was double that of the other two isomers. *O*-cresol is known to be the most recalcitrant of the cresol isomers (Boyd *et al*., 1983; Smolenski and Sulfita, 1987; Flyvbjerg *et al.,* 1993; Spence *et al*., 2001), it is found in waste run off from gas works, coal tar plants and wood treatment plants. It readily enters the groundwater environment; it is known to be hazardous to aquatic life and toxic to humans.

Xylenols (dimethylphenols) are compounds with a similar structure to cresols, but with the addition of an extra methyl group to the aromatic centre (Figure 1.3). They are in the Short Chain Alkylphenols (SCAP) group of compounds. Xylenols like cresols readily find their way into the natural environment as they are constituents of crude oil and can be found in many

household and industrial chemicals. The extra methyl group decreases the solubility of the compound and is thought to increase the toxicity of that compound. A number of studies have been carried out to establish the toxicity of alkylphenols (Devillers, 1988; Kahru *et al.,* 1999; Acuña-Argüelles *et al*, 2003), these studies indicate that xylenols are more toxic than cresols, although contradictory evidence has also been published (Kahru *et al.,* 2000). A study by Kim *et al.,* (2003) gave results, which showed phenol to exhibit the same toxicity as *o*-cresol when tested using a *Daphnia magna* bioassay for $EC_{50,}$ although other tests conducted in this study showed o-cresol to be more and less toxic than phenol depending on the assay used. The testing methods used in such studies notably caused differences in results. Kahru *et al.,* (2000) studied the toxicity of phenolic compounds including phenol, p-cresol and 2,4-Dimethylphenol using a number of different toxicity testing kits and obtained mixed results (Table 1.3), from this table it is clear that the toxicity of alkylphenols changes with the toxicity test.

Table 1.3 Toxicity L(E)C50 mg/L tests for various phenolic compounds (adapted from Kahru *et al.,* (2000).

	Photobacteria V. fisheri	*Crutaceans Daphina*	*Protazoa Tetrahymea*	*Algae Selenastrum*	Toxicity ranking
Phenol	19	10	520	244	toxic
p-cresol	1	6.5	90	188	very toxic
2,4-dimethylphenol	3.7	5.4	70	20	toxic
2,3-dimethylphenol	5.3	11	190	50	toxic
3,4-dimethylphenol	0.39	6.3	90	53	very toxic

The compound utilised in the present study was 2,4-Dimethylphenol (DMP) a compound similar to *o*-cresol, but with the addition of a methyl group at position 4 of the aromatic ring (Figure 1.3.) DMP is found in crude oil and is a product of petroleum and crude oil fractionation (Acuña-Argüelles et *al*., 2003). In a study of coal tar liquefaction plumes in groundwater Harrison *et al.,* (2001) recorded levels of DMP of 0.82mM, which, with the exception of the cresol isomers, represented the highest concentration of contaminating alkylphenols.

1.5 Toxicity

Although there are some data on the toxicity of SCAP compounds, only 30% of the 35 SCAP compounds have had their toxicity assessed. It is generally considered that with the addition of a methyl group the toxicity of the

compounds increases. Table 1.3 shows the toxicity of some SCAP compounds. Kahru *et al*., (1999) did not, however, test 2,4-dimethylphenol.

Table 1.4: Toxkit microbiotests data (Kahru *et al*., 1999)

Compound	EC_{50} (mg/l)	compound	EC_{50} (mg/l)
Phenol	97.3	2,3-dimethylphenol	41.2
o-cresol	51.8	2,6-dimethylphenol	29
m-cresol	83.8	3,4-dimethylphenol	6.1

Although it is generally considered that with the addition of a methyl group the toxicity of a compound increases, Devillers (1988) noted that the acute toxicity of alkylphenols on the water flea (*Daphinia magna*), as determined by IC_{50} values (50% inhibition) gave different results than would be expected. Devillers (1988), noted that cresols were more toxic than phenol, but dimethylphenols did not appear to have a significantly higher toxicity than cresols and trimethylphenols were, in fact, less toxic. The experiment concluded that the number and position of the methyl groups attached to phenol had no direct relationship to their toxicity to the water flea. These results could be due to the different metabolic pathways of the chemicals, or to other factors such as differential solubilities. According to Ruey-Shin and Shang-Yuan (2006) phenolic compounds, including cresols, are potential carcinogens and can reduce enzyme activity and in turn cause fatalities amongst fish at concentrations as low as 5-25mg/l.

The study by Holmes *et al.,* (2002) utilised three stage continuous flow multi stage systems to analysis the effects of sulphate and *o*-cresol concentration on the anaerobic bacterial groups. The systems allowed for the spatial separation of the bacterial groups, therefore allowing the effect of *o*-cresol on each bacterial group to be observed. The inocula for their study were landfill refuse and hexanoate was the carbon source. It was shown that o-cresol concentrations of 4 mM had an effect of sulphate reducing bacteria. Concentrations ≥10mM of *o*-cresol were shown to inhibit hydrogenotrophic methanogens, and it was shown that acetoclastic methanogens were only partially inhibited at 10mM indicating that they were more resistant too o-cresol.

Both the compounds of interest are substituted phenols. According to Ramos *et al.,* (1998) this groups them in the polar narcotic group of toxicants. Narcosis is a non-specific reversible state of arrested activity, which is due to contact with a wide variety of organic compounds (Veith and Broderius 1990). Polar narcotics, like non-polar narcotics, disrupt the phospholipid functioning membrane of the bacterial cells, causing death or inhibition of function. Polar narcotics have a greater toxic effect than non-polar due to them having the ability to form hydrogen bonds with the membrane. Polar narcosis is linked to compounds hydrophobicity and hydrogen bond donor activity (Verharr *et al.,* 1992; Lin *et al.,* 2004). Therefore an increase in hydrophobicity increases the toxicity of a compound, from this it can be hypothesised that the less soluble DMP is more toxic than *o*-cresol.

Despite the varying opinions on the toxicity of alkylphenols they are still at least as toxic as phenol and therefore due to the fact that they have fairly

high solubilities and toxicities many magnitudes below this, this study is environmentally relevant. Boyd *et al* (1983) stated that phenols, as a group, are highly toxic, although limited information exists on the carcinogenicity of most phenols.

Phenolic compounds including alkylphenols are known to be toxic to plant and animal life. *O*-cresol and 2,4-dimethylphenol are both known to be toxic and both are packaged with hazard labelling. The US EPA has given SCAP compounds an environmental concern level of 0.02 μg/l to aquatic life (Suater and Licha, 2002). If the compounds enter drinking water it can no longer be considered safe to consume. This could really cause a problem in countries where drinking water is a scarce commodity or in developing countries, which are not aware that some of the practices they employ, allow these compounds to enter the environment. According to Piver (1993) groundwater contamination is a concern due to the toxicity towards humans and the ecotoxicity. Drinking water containing phenolic compounds, at levels of 2.0μg/l will taste and smell objectionable (Ruey-Shin and Shang-Yuan (2006).

Not much is known about the effect these compounds exhibit on humans (Suater and Licha, 2002), and it would appear that to obtain a toxic effect on a human, large quantities of these compounds would be required, although these compounds can enter the human body by different means, for example, via skin, inhalation and ingestion.

Although most of these studies only analyse the effect of one alkylphenol, it is important to note that in contaminated groundwater there will be more

than one alkylphenol present, in addition to other contaminants. It is therefore important that the combined effects of these compounds should be studied. It may be that when there are two or more alkylphenols present their combined toxicity and inhibitory effects may be antagonistic, additive or even synergistic.

- antagonistic effects- if this occurs the effects of the individual chemicals may cancel each other out ,thus causing less of an inhibitory effect
- additive effects- the combined effect of the two chemicals is the sum of the effect of each individual chemical
- synergistic effect- is a response greater than the sum of the combined effects of the chemicals.

Previous studies have shown that polar narcotics, when combined, have a predictable additive toxic effect (Verharr *et al.,* 1992; Lin *et al.,* 2004). Although it may be possible for the breakdown of one compound to inhibit the breakdown of another, which in turn could increases toxicity.

1.6 Degradation

There has been little research into the eventual fate of alkylphenol compounds in the environment. A study by Kahru *et al*. (2002) concluded that alkylphenols are readily biodegradable in a soil environment. However this study only examined aerobic degradation, and did not address anaerobic degradation. The anaerobic degradation of all three cresol isomers has been shown in lab and field studies (Smolenski and Sulfita, 1987; Fang and Zhou, 1997; Londry *et al*., 1997; Spence et al., 2001) and it is thought that other

alkylphenols can be degraded under anoxic conditions. Studies carried out on the anaerobic degradation of cresols have found o-cresol to be the most recalcitrant of the three isomers (Smolenski and Sulfita 1987; Flyvbjerg *et al.*, 1993) with some experiments reporting no significant degradation (Boyd *et al*., 1983), which, could be due to longer lag times than the experiments allowed, (Flyvbjerg *et al*., 1993; Spence *et al*., 2001). According to Ruey-Shin and Shang-Yuan (2006), phenol and cresols are recalcitrant and highly stable toxic compounds and the fact that they are aromatic makes them more resistant to natural biodegradation.

Previous work on the degradation of these chemicals has shown biodegradation, although not enough research has been done on the biodegradation of organic chemical mixtures in an anoxic sub surface environment. It is thought that mixtures of pollutants are more frequently found in groundwater than single organic pollutants. Mixtures of pollutants makes biodegradation more complex, e.g. degradation of one chemical may be inhibited by another in the mix or the bi-products of one chemicals degradation may inhibit further degradation. In terms of conditions, chemical and physical, these may not be the same for the degradation of similar pollutants.

As mentioned previously, the groundwater environment contains bacterial consortia, which work in conjunction to break down organic compounds. 2,4-Dimethylphenol and *o*-cresol may greatly reduce the efficiency of this activity, therefore allowing other hazardous chemicals to enter the drinking water environment. In underdeveloped countries that do not have effective means of sterilising drinking water this could be potentially serious.

1.7 Method of Study

To investigate the effects of these two compounds, anaerobic microbial batch cultures were set up. In order to determine the effects of chemicals on bacterial metabolism various analytical methods were employed e.g. gas, high performance liquid and ion chromatography. Analysis of sulphate concentration was carried out in order to assess the inhibition of the sulphate reducing bacteria, while methane production, volatile fatty acid production and utilisation, and pH were also analysed to identify any inhibitory effects of the compounds on the bacterial consortium.

The use of batch cultures provided a guide to the minimum inhibitory concentrations (MIC) of the chemicals and, if an appropriate concentration range was initially selected, the information to determine the inhibitory range, 0- 100% inhibition, was found.

The batch cultures allow the analysis of the effects of increasing concentrations of *o*-cresol and DMP. It has been previously reported that the addition of the xenobiotic at the start of an experiment has a stronger effect on some bacterial types than it does after the establishment of these groups for example the methanogenic bacteria (Sulisti 1994). This can be seen in Section 5.3.2 where it was observed that an established methanogenic bacterial community has a higher tolerance to *o*-cresol than one where *o*-cresol is added at the beginning of the experiment.

It is important to note that in this experiment the *o*-cresol and DMP were added to their respective culture sets at the beginning of the study to enable the analysis of the effects they exhibit on a mixed population as a whole

rather than the individual bacterial groups. This allows the analysis of so called knock-on effects that occur due to the inhibition of one bacterial group and the effects this has on the other bacterial groups in the community. As mentioned in Section 1.3 a consortium of different anaerobic bacterial species working in a synotrophic relationship are needed for the complete mineralization of a carbon source, compared to one aerobic species. Therefore if one of these bacterial groups becomes inhibited it can affect the degradation process in turn causing indirect inhibition of other bacterial groups.

Batch cultures allow the analysis of the effects o-cresol and DMP have on the whole consortium whether directly or indirectly.

The main groups of bacteria studied in this experiment were SRB, acetogens, acetoclastic and hydrogenotrophic methanogens.

Due to the nature of these batch cultures, the effects of the compounds on individual components of the bacterial consortium could not be identified, although by analysing and combining the results gained from the various analytical techniques, certain hypotheses were drawn (chapter 3). Therefore to identify the specific bacteria affected and at what concentrations of these compounds, continuous flow three-stage systems were set up, a schematic diagram of a three-stage continuous flow system can be seen in Figure 1.4.

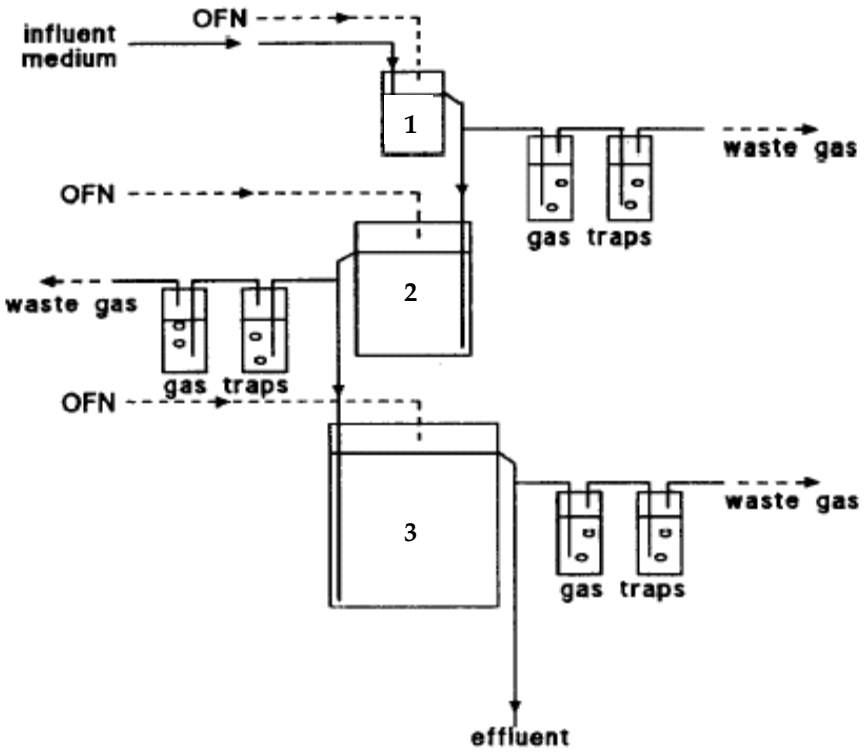


Figure 1.4: A continuous flow, three-stage system (James *et al*., 1998).

These systems (Figure 1.4) allow the segregation of the different groups of anaerobic bacteria into the individual vessels giving them their own habitat domain but still allowing metabolic interactions between groups i.e. overlapping activity domains (Holmes *et al*., 2002). The different vessel sizes, and the resultant differences in dilution rates, enable segregation on the basis of individual growth rates. Analysis of metabolic products and removal of electron donors and specific electron acceptors confirms the segregation of individual groups. The system thus enables analysis of the effects of *o*-cresol and 2,4-Dimethylphenol on specific bacterial groups such as methanogens and sulphate reducing bacteria, and can be used to analyse the implications of the inhibition of one group of bacteria on the bacterial consortium.

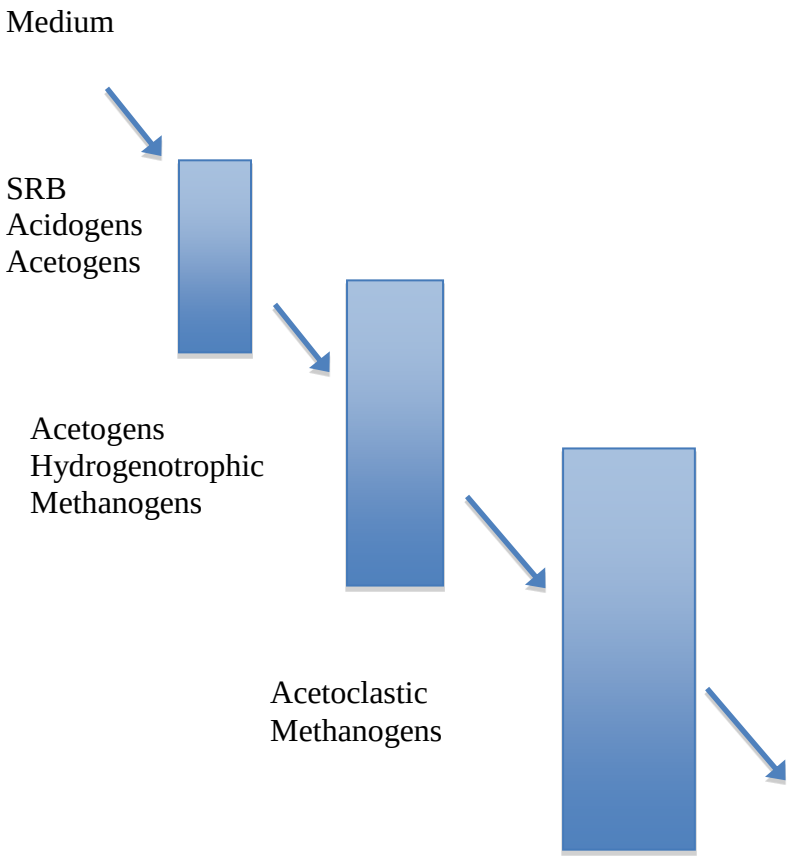


Figure 1.5 Spatial separations of the predominant bacteria in the continuous flow system.

Figure 1.5 shows the separation of the predominant bacteria that will have their activities analysed using the system initially developed in the study by Sulisti (1994). In the study by (1994), it was observed that sulphate reducing bacteria (SRB) established in the top vessel, the acetogenic and hydrogenotrophic bacteria in the second vessel, and lastly the acetoclastic bacteria are found in the third vessel. Further studies conducted by James *et al.*, (1998) and Holmes *et al.*, (2002) showed this segregation of the bacterial groups.

The system set-up enables the contamination of a specific vessel, i.e. if the bottom vessel was exposed to the chemicals first then the specific effect and

inhibitory effect of the chemicals on the bacteria inhabiting the bottom vessel can be determined and studied without affecting the bacterial habitats in the vessels above. After the work has been completed on the lower vessel the contaminants are introduced to the vessel sequentially above.

The potentially toxic chemical may also be introduced initially into the top - vessel 1- which would enable the knock on effect of the compound on all the bacteria to be monitored. For example if the sulphate reducing bacteria in vessel 1 were inhibited then the output from the top vessel would contain residual substrate and intermediates with the implication that the carbon and energy flows in vessels 2 and 3 may be affected.

Another benefit of this type of system is that it can be used to monitor the degradation of complex wastes such as diesel oil (Wimpenny *et al*., 1993). In a similar fashion, the possible degradation of *o*-cresol and 2,4-dimethylphenol may be studied. The multi-stage system allows control of different conditions in each vessel e.g. pH and temperature, and it also allows the user to add specific chemicals or enrichment medium to each individual vessel separately (Parkes and Senior, 1988). This property allows for the possible enhancement of the biodegradation of the two alkylphenols, since if the specific conditions and bacterial populations that achieve this are identified, then appropriate electron acceptors and substrates can be added.

The reason that microcosms and batch cultures containing mixed cultures were used, as opposed to using pure cultures, was to enable the study of the effects the contaminants would exhibit on the complex synotrophic relationships of mixed bacterial environments, like those found in the groundwater environment.

To further understand the anaerobic growth consortia and the segregation created by the multi-stage systems, molecular biology techniques may be applied. Anaerobic growth consortia contain many different types of bacteria and archaea. If bacteria or archaea were discovered within the consortia that were resilient to the toxic effect of o-cresol or DMP, it would be beneficial to isolate these organisms to further understand their resistance; this is where the problems arise. Amann *et al.,* (1995) states that it is only possible to pure culture 1% of bacteria from environmental samples (Table 1.5), therefore it may be impossible to isolate the specific bacteria in pure culture. Molecular techniques would also allow a more thorough investigation into the population shifts of the consortium without the problems associated with pure cultures. The molecular techniques would enable the identity of the specific bacteria that are involved in specific processes within the consortia to be discovered.

Table 1.5 % culturability of bacteria and archaea taken from different environments (Amann *et al.*, 1995)

Habitat	Culturability (%)
Seawater	0.001-0.1
Freshwater	0.25
Mesotrophic Lake	0.1-1
Unpolluted estuarine waters	0.1-3
Activated sludge	1-15
Sediments	0.25
Soil	0.3

The use of selective enrichment cultures also fail to get the exact conditions some bacteria in a consortium need for growth (Muyzer *et al.*, 1993). In recent

years advances in molecular biology techniques have enabled further insight into the diversity of these bacterial consortia (Ovreas *et al.,* 1997; Wise *et al.,* 1997; Davies *et al.,* 2004). The use of 16S rDNA with denaturation gradient gel electrophoresis (DGGE) and DNA sequencing are molecular techniques which enable the study of the different bacterial and archaeal groups in mixed bacterial populations and also allow for the analysis of population shifts within bacterial communities. According to Kawaii *et al.,* (2002) DGGE is a widely used technique, which enables the identification of bacterial species without their isolation. DGGE is a rapid diagnostic tool to discover the constituents of a bacterial population (Muyzer *et al.,* 1993). The extraction and amplification of 16S rDNA followed by the separation of the different bacteria species DNA using DGGE allows for further understanding of bacterial populations within mixed cultures. These molecular techniques enable the study of population shifts in the bacterial communities.

By utilising these techniques the different bacterial groups that establish in the vessels of multi-stage systems can be studied allowing further investigations into the populations present and enables the further investigation into the direct effects the chemicals *o*-cresol and DMP exhibit on the different bacterial groups, allowing further investigation into bacterial tolerance.

The use of universal bacterial and archaeal PCR primers is a relatively new idea although many studies have been conducted which employ these methods (Bruggemann *et al.,* 2000; Gonzalez *et al.,* 2003; Sigler *et al.,* 2004). Universal primers allow the polymerase chain reaction to amplify all of the bacteria and archaea in a consortium. These universal primers target the

conserved regions on the 16S rDNA gene; thus allowing the amplification of the variable regions within these conserved regions. As bacteria and archaea have evolved, all have kept conserved regions, which are universal; therefore the use of the universal primers enables the amplification of all the bacteria and archaea in a sample. By coupling this technique with a denaturation gel electrophoresis DGGE, the amplified species can be separated which allows further study and identification of the bacteria and archaea present in the consortium. These methods will also allow for further investigation of the effects that o-cresol and DMP have on specific bacterial strains within the consortium.

With the amplification of all the bacteria and archaea in a sample, the samples can then be subjected to electrophoresis on a denaturation gradient gel. The denaturation gel contains a denaturation gradient of urea formamide; this gradient denatures the G-C bonds within the DNA duplex of the sample. As the sample is run on the gel the bacteria with the least G-C content become fixed to the gel and those with more continue down the gel and eventually stop at different positions due to the increased denaturation gradient. The DGGE allows for the separation of all the bacteria in the sample. This means that individual bands seen on the gel are individual bacterial or archaeal species. These bands can then be excised and sequenced allowing the naming of the specific bacterial species.

It must although be noted that the use of PCR DGGE is a rapid and important diagnostic tool it does have its drawbacks. Firstly PCR does not discriminate between live and dead bacteria (Nocker and Camper 2006) and it is possible for DNA persistence up to 3 weeks after cell death (Nocker and Camper, 2006; Nocker *et al*., 2007). This therefore means that when studying

the effect toxic chemicals have on a bacterial population the use of PCR may yield false results due to dead bacterial DNA being amplified. DGGE does not show all bacteria present in a consortium, it has been stated that bacteria which constituent a low percentage of the total community will not be detected by DGGE (Muyzer *et al*., 1993; Dar *et al*., 2005). Although there are negative points to these techniques they still provide a valuable tool for the analysis of bacterial consortia and in this study will help to indicate population differences between the different vessels of the systems.

1.8 Objectives

1. To assess the toxicity of *o*-cresol and 2,4-dimethylphenol on bacterial growth consortia in an anaerobic environment.
2. To assess the toxicity of *o*-cresol and 2,4-dimethylphenol on individual groups of bacteria within a consortium utilizing continuous flow three-stage systems.
3. To utilise molecular techniques to obtain a clearer picture of population shifts within the anaerobic consortium.
4. To determine the toxicity of the mixture of the two compounds.

Chapter 2

Material and Methods

2.1 Materials

2.1.1 Chemicals

The chemicals used throughout the experiments were all purchased from VWR BDH laboratory supplies (Leicestershire, U.K.), with the exception of NH_4Cl, cellobiose, 2,4-dimethylphenol (DMP) and *o*-cresol that were supplied by Sigma-Aldrich Company Ltd. (Dorset, U.K). All were "AnalaR" grade except for formic acid which was "AristaR" grade, and acetonitrile and 85% orthophosphoric acid which were "HiPerSolv for HPLC" grade.

The buffers used in the standardisation of the pH meter were made from Tri-check buffer capsules (Sigma-Aldrich, Dorset, UK).

Oxygen free nitrogen (OFN), methane, air, oxygen and helium were all obtained from the British Oxygen Company (BOC, Glasgow, U.K.).

2.1.2 Growth Media

The mineral salts medium, employed for both the batch cultures and the continuous flow multi stage systems was described by Holmes *et al* (2002). The medium contained (gl^{-1} in distilled water): K_2HPO_4, 1.5: $NaH_2PO_4.2H_2O$, 0.85; NH_4Cl, 0.9; $MgCl_2.6H2O$, 0.2; $NaHCO_3$, 0.5; Na_2CO_3, 0.2; trace elements, 1.0ml; trace minerals, 1.0ml; nickel solution (1mmol l^{-1}), 1.0ml; vitamin solution, 1.0ml.

The stock trace elements solution contained (mgl^{-1}): NaCl, 9000; $FeCl_2.4H_2O$, 1500; $MnCl_2.4H_2O$, 197; $CoCl_2.6H_2O$, 238; $CuCl_2.2H_20$, 17; $ZnSO_4$, 287; $AlCl_3$, 50; H_3BO_3, 62; $NiCl_2.6H_2O$, 24.

The stock trace mineral solution contained (mgl^{-1}): $NaMoO_4.2H_2O$, 48.4; $NaSeO_3H_2O$ (31% Se), 2.55; $Na_2WO_4.2H_2O$, 3.3.

The vitamin solution contained (mgl^{-1}): biotin, 10; *p*-aminobenzoic acid, 19; a-liponic acid, 20; folic acid, 10; pyroxidine HCl, 20; thiamine HCl, 20; riboflavin, 30; nicotinic acid, 50; D(+) Ca-pantothenate, 30; cyanocobalamine, 20. In addition 5.02mM ($1720mgl^{-1}$) of cellobiose was added as a carbon source.

Anaerobic batch culture media were not sterilised. The medium used for the continuous flow systems was sterilised by use of a Whatman membrane filter (cellulose nitrate, WCN, 0.2 μm pore size, 142mm diameter; Fisher Scientific, Leicestershire, U.K.) attached to a 142mm stainless steel holder (Sartorius Gottingen, Germany).

2.1.3 Inocula

Batch cultures and the continuous flow multi-stage systems were inoculated with return activated sewage sludge, to ensure bacterial activity. 30 litres of sludge was collected from the Paisley sewage treatment plant, Glasgow, United Kingdom. On return to the laboratory the sludge was stored in the dark at 4°C overnight. The sludge was allowed to settle for 24-48 hours, allowing the solid sludge particles to sink to the bottom giving an upper liquid layer. The upper liquid was discarded and the sludge was stored at 4°C until it was needed, for a maximum of 6 months. It was thought that a reduction in bacterial activity might occur if the sludge was stored longer than this.

The volume of inocula used per experiment was 50ml for a 250ml total batch culture. The inocula for the multi stage systems was 125ml of a 250ml batch

culture that had been incubated for 30 days to ensure the different groups of bacteria had enough time to establish. Immediately before the inocula were used in any of the experiments, the container holding it was shaken to mix the bacterial population. Both the batch culture and the multi-stage system experiments were carried out at 30°C.

2.1.4 Sample preparation

1ml samples were extracted using 1ml sterile syringes from both the batch culture and system vessels. These samples were then centrifuged for 15 minutes at 14000rpm (20 min. for HPLC analysis of 2,4-DMP and *o*-cresol concentrations). The supernatant was then removed and transferred into 1.5ml polypropylene Eppendorf tubes and used to determine pH and sulphate concentration, 0.45 ml was put into screw cap glass vials with the addition of 0.05ml of formic acid for use in volatile fatty acid (VFA) analysis. Samples were analysed immediately or held at -20 °C until needed.

2.2 Analytical methods

2.2.1 Determination of pH

The pH of the batch cultures and the vessels in the systems was obtained using a 662-1767 semi-micro pH electrode (VWR, Leicestershire, U.K.) attached to a 3010 pH meter (Jenway (Essex, U.K.). pH buffered solutions of 4.0, 7.0 and 10 enabled the calibration of the instrument.

2.2.2 Determination of sulphate

The supernatants from the samples were diluted with distilled water (batch samples 1:25 dilution, system samples 1:4 dilution). The diluted samples were loaded into a DX-120 ion chromatograph (Dionex, Surrey, U.K.) with an internal ASRS-ULTRA suppressed conductivity detector, which used an eluent containing 0.5M $NaHCO_3$ and 0.5M Na_2CO_3. The injection volume was 25µl and the flow rate was 1.5ml/min. Before running samples, standards (containing 30mgl^{-1} of both anions) were run to enable quantification of the amount of sulphate present in the samples. Samples and standards were run according to the manufacturer's protocol.

2.2.3 Determination of methane in the batch reactions

Methane was determined using the method of Nitayapat (2003). Using a Hamilton gas tight syringe (Bonaduz, Switzerland), 50µl of gas from the headspace above the cultures was injected into a Perkin-Elmer (Milan, Italy) 8700 Gas Chromatograph (GC) equipped with a flame ionisation detector with the flow rate of the carrier gas (OFN) set at 50ml min^{-1}. The GC was fitted with a stainless steel packed column containing (2mm i.d.) 5% neopentyl glycol sebacate + 1% H_3PO_4 on Chromosorb W-AW (80-100 mesh) (Speck Analytical, Alva, UK). Temperatures of 80, 200, and 210°C were used for the oven, injector and detector respectively. Three 50µl injections were made from each sample and the results were compared with the total peak area obtained by injecting five 10µl portions of pure methane (CP grade, BOC). Atmospheric pressure and the temperature of the laboratory were noted during the analysis. Concentrations of methane contained in the samples at standard temperature and pressure were determined by

comparing mean peak areas of samples and standards after the construction of a standard curve.

2.2.4 Determination of short chain volatile fatty acids (VFA)

The method of Nitayapat (2003) was used to determine VFA's. The sample was prepared as in Section 2.1.4. Samples were thawed and 1µl of the prepared samples were injected into the Perkin Elmer 8500 Gas Chromatograph equipped with a flame ionisation detector with the flow rate of the carrier gas (OFN) was set at 50ml min^{-1}. The GC was fitted with a 2m stainless steel packed column (2mm i.d.), which contained, 5% neopentyl glycol sebacate + 1% H_3PO_4 on Chromosorb W-AW (80-100 mesh). The temperature gradient of the oven was as follows; after sample injection the temperature was kept at 108°C for 2 min, from this point it increased at 25 °C /min until the oven reached 120°C, for 0.2min the temperature stayed at 120°C then increased at a rate of 8°C/min to 145°C, after 0.2 min at 145°C the temperature was increased by a further 25°C/min until a final temperature of 165°C was reached. The injector and the detector were kept at constant temperatures of 220 and 210°C respectively. The peak areas observed from the samples were compared with those obtained from injecting 1µl of an acidified mixture containing 10mM of each fatty acid. A computer linked to the GC contained the Clarity chromatogram package which enabled analysis of the peak areas and allowed interpretation of the results. In order to test the validity of the results standard solutions were run on an intermittent basis during the assessment of the VFA samples

2.2.5 Determination of o-cresol, phenol and 2,4-dimethylphenol

1ml samples were centrifuged at room temperature for 20min at 14000rpm in a Sigma 2-16K centrifuge; 0.35ml samples of the supernatant were placed into a Gilson (Anachem Ltd., Luton, U.K.) 234 Auto injector, which injected 20µl aliquots into a 4.6mm x 150mm stainless steel column containing a Gemini C18 (5µm) (Phenomenex, Cheshire, U.K.). The column was placed in a water bath which was then covered in order to keep a constant temperature of 35°C. The column was connected to a Gilson UV/VIS 119 detector set at 230nm. Elution of the HPLC column was accomplished using Gilson 305 and 306 pumps, which pumped a concentration gradient of 100% acetonitrile in 0.01M phosphoric acid at a flow rate of 1.5ml min^{-1}. The chromatographic conditions were as follows:

Column: Gemini C18 (5µm)

Flow Rate: 1.5ml/min

Column Temperature: 35°C

Injection Volume: 10µL (1nmol / peptide column loading)

Mobile Phase: A: 0.01M Phosphoric acid

Mobile Phase B: Acetonitrile

Detection: UV 230nm

Run Time: 10 min.

Re-equilibration time: 5 min.

Gradient:

Time	%A	%B
0 min	80%	20%
7.5 min	55%	45%
8.0 min	55%	45%
10.0 min	30%	20%

This method is a modified version of the Chrompack phenols and substituted phenols application note method obtained from the CP-SCAN view CD-ROM (Chrompack, Varian, U.K.), the modifications were made after analysis of standards, and allowed the reduction of run time, due to the compounds of interest having retention times less than 10 minutes. Calibration curves for 2,4-DMP and *o*-cresol (0-4.9 mM for 2,4 DMP and 0-14mM for *o*-cresol) were created using known concentrations of these substances and plotting them against peak areas. The samples were run using the calibration graphs (Figures 2.1 and 2.2) to give the concentrations of the three compounds. This method was used to check the concentrations of DMP and *o*-cresol throughout the batch and multi-stage systems studies (Chapters 3 and 4).

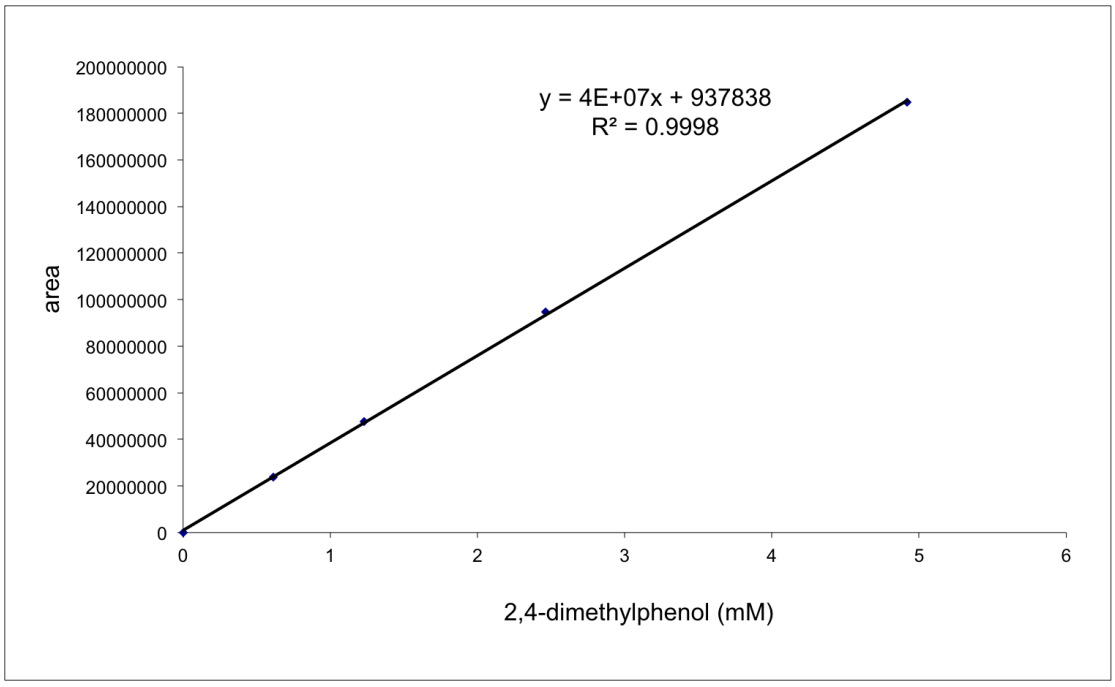


Figure 2.1 HPLC calibration curve for 2,4-dimethylphenol

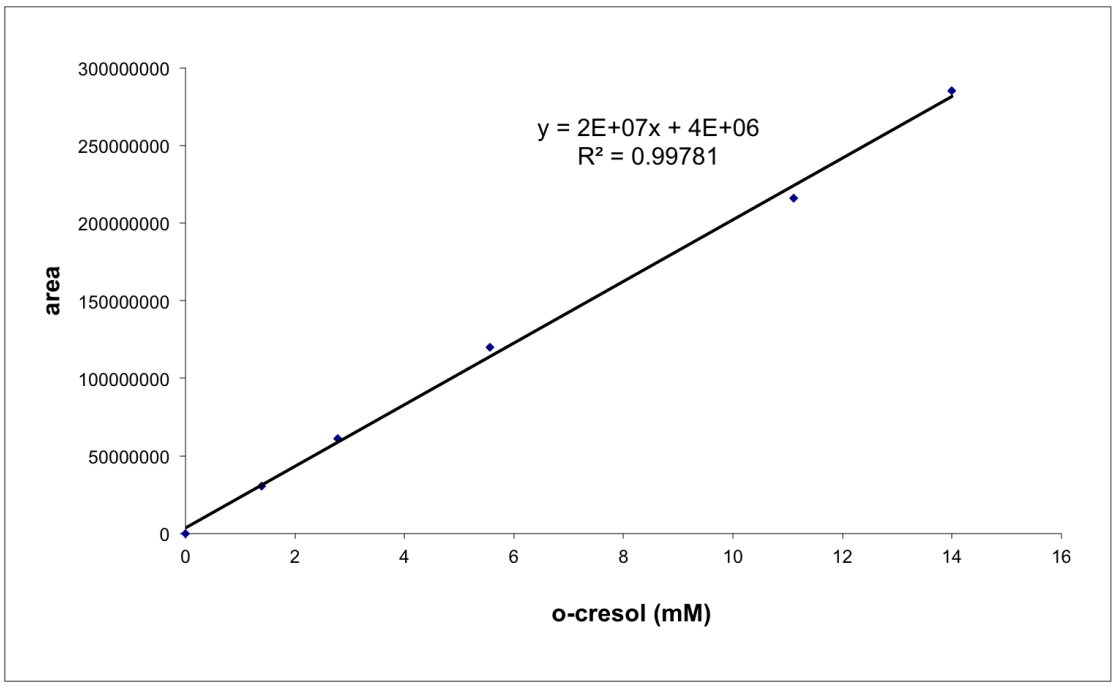


Figure 2.2 HPLC calibration curve for *o*-cresol

2.2.6 Determination of solubilised methane from the continuous flow systems.

This method was developed by Watson-Craik and Senior (1989). A standard curve (Figure 2.3) was plotted using solubility of methane in water under 1atm of methane, at temperatures 0-70°C. Standards were prepared by sparging methane (CP grade, BOC) through 10ml of distilled water, until completely saturated (30min). The temperature of the water at the end of the sparging period was noted. Bijou bottles sealed with sub-seals were evacuated for 30 seconds using a vacuum pressure pump (Laboport, Type N86 KT18, KNF Neuberger, New Jersey, U.S.A), to these 1ml aliquots of the methane saturated solution were injected.

Samples of 1ml were extracted from each vessel in the multi-stage systems and injected as above into evacuated bijou bottles, hypodermic needles were then used to equilibrate the internal negative pressure of the bottles to atmospheric, and the needles were then removed.

The methane concentration of the bijou bottles gas phase, for the standards and the samples, was determined by injecting 50µl (in triplicate) samples into a Perkin-Elmer 8700 GC equipped with a flame ionisation detector. Oxygen free nitrogen at 35ml/min was used as a carrier gas and the column mentioned in Section 2.2.4. The temperatures of the injector, oven and detector were 200, 80 and 210°C, respectively. The concentration of dissolved methane produced by the vessels in the system in mmol/l was determined by comparison with the standard curve shown in Figure 2.3.

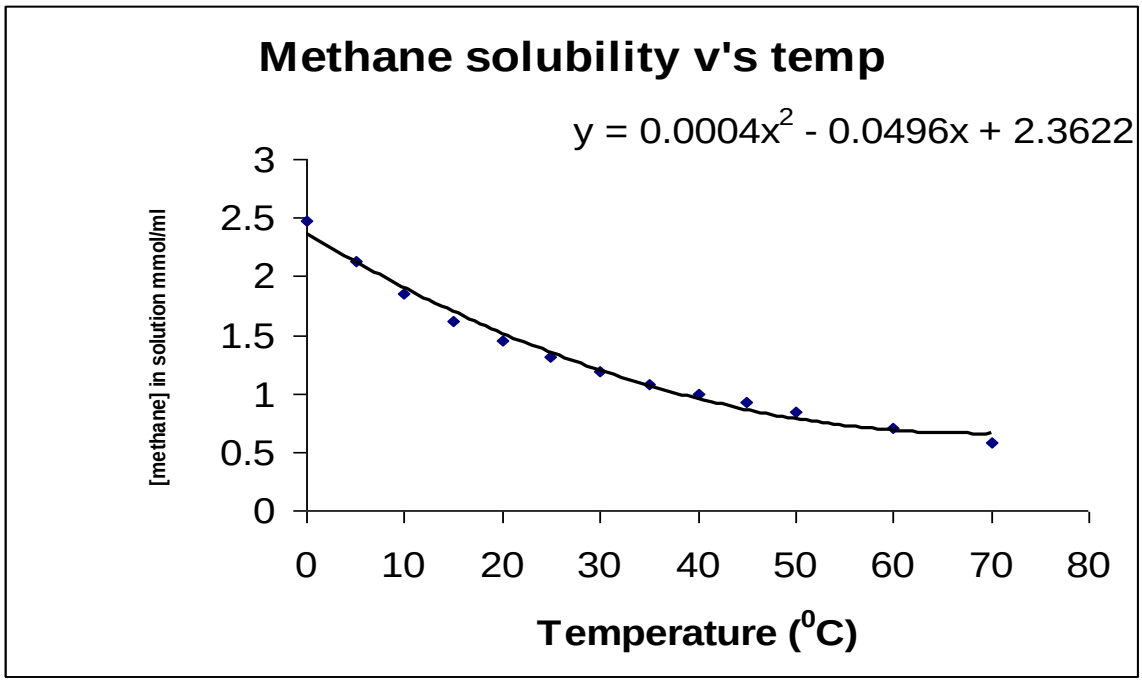


Figure 2.3 Methane water solubility versus temperature

Methane concentration was then calculated by the following equation:

C (mmol/L) = (y/ Average STD peak area)*Average peak area of samples

2.3 Molecular Biology Materials

2.3.1 Materials

2.3.1.1 Media and Buffers

All chemicals, unless otherwise indicated, were supplied by Sigma-Aldrich (Gillingham, UK). All solutions were prepared as follows:

2.3.1.2 50 × Tris Acetate EDTA buffer (TAE)

242g Tris base, 18.6g EDTA, and 57.1mL glacial acetic acid were added to 900mL of distilled water. The pH was adjusted to 8.0 with 5M NaOH and the volume was adjusted to 1 litre using distilled water. The solution was autoclaved at 121°C for 15 min.

2.3.1.3 Tris EDTA Buffer (TE)

1L of 10mM Tris-Cl (pH 8.0) with 1mM EDTA (pH 8.0) was prepared in distilled water and autoclaved at 121°C for 15 min.

2.3.1.4 10 × Phosphate Buffered Saline (PBS)

80g NaCl, 2g KCl, 11.2g Na_2HPO_4, and 2g KH_2PO_4 were added to 900mL of distilled water. The volume was adjusted to 1 litre with distilled water. The solution was autoclaved at 121°C for 15 min.

Chapter 3

O-cresol and 2,4-dimethylphenol contaminated batches

3.1 Introduction

In this study two sets of batch cultures were set-up to establish the toxic effects exhibited by *o*-cresol and 2,4-Dimethylphenol on sulphate reducing bacteria, acetogenic bacteria and methanogens.

3.2 Experimental method

Batch culture bottles in triplicate were set up. Batch cultures contained concentrations of *o*-cresol between 1.85mM and 11mM and 2,4-dimethylphenol (DMP) between 0.41mM and 4.1mM. The concentrations were chosen as they were suspected to give a range of effects from no inhibition to complete inhibition. The *o*-cresol concentrations were based on previous work by Holmes et al., (2002) and the DMP concentrations were chosen after an initial scoping experiment on inhibition of sulphate reduction using DMP concentrations of 0.08, 0.16, 0.33, 0.49, 0.62, 0.82 and 1.23mM (Appendix 1, Figure I.1). All cultures were supplemented with SO_4 (4.2mM) and stored at 30°C and in darkness. Alongside the cultures containing *o*-cresol and DMP, control cultures were setup in the exact same way with the omission of the phenolic contaminant. The batch culture experiments were run at different times with the o-cresol batch experiment being first, followed by the DMP experiment; therefore separate control cultures were needed for both experiments.

Analysis of sulphate, VFA and methane concentration, as well as pH was carried out on all cultures as described in chapter 2. HPLC analysis of *o*-cresol and DMP concentration according to Section 2.2.5 at the start and end of the experiment, (the o-cresol experiment lasted 47 days and the DMP experiment lasted 41 days, these differences were due to the differences in

the activity of the inocula), was carried out to ensure there was no degradation, no change was noted in concentration

3.2.1 Preparation of batch cultures containing return activated sludge

Two sets of batch cultures were prepared one for the analysis of *o*-cresol and the other for DMP analysis. The cultures were prepared in triplicate in 500ml Gilco bottles, which were sealed with rubber suba-seals (Fisher Scientific UK Ltd, Loughborough, UK). Each bottle contained 200ml of growth medium (Section 2.1.2) and 50ml of the return activated sewage sludge inocula (Section 2.1.3), giving a final volume of 250ml. The batch cultures were also supplemented with sulphate (4.2mM). A previous study into the effects of *o*-cresol on bacterial communities obtained from landfill refuse by Holmes *et al.*, (2000) used sulphate concentrations of 3.5mM. It was thought that by increasing the sulphate concentration to 4.2 mM, an abundance of sulphate would be present. To the batches, set concentrations of *o*-cresol (1.85mM, 3.7mM. 4.6mM, 5.6mM, 7.4mM 9.3mM and 11mM) or 2, 4-DMP (0.41mM, 0,82mM, 1.23mM, 1.64mM, 2.46mM, 3.28mM and 4.1mM) were added, separately. After the bottles were sealed they were then flushed of oxygen using oxygen free nitrogen OFN (BOC gas, U.K.) thus creating anoxic conditions. The cultures were then incubated in the absence of light at 30°C to ensure no activity from phototrophic bacteria, which could potentially lead to false results.

Cellobiose 5.02mM (Section 2.1.2) was used as the growth substrate in these experiments as it is a soluble hydrolysis product of cellulose (Shi and Weimer, 1997). Cellulose is readily found in the environment, but to use it would have increased the timescale of the experiment substantially. The use of cellobiose was to enable the emulation of environmental substrate

conditions found in the groundwater environment, cellulose can be degraded to cellobiose, which in turn will be, metabolised to smaller VFA e.g. acetate, propionate and butyrate. Hemme *et al., (*2010) suggests that cellobiose may permeate into groundwater from adjacent soils.

3.3 Results and Discussion

3.3.1 Sulphate reduction *o*-cresol

Sulphate concentrations in the batch cultures were measured using ion chromatography (Section 2.2.2). From Figure 3.1 it can be seem that the initial sulphate concentrations in all the batch cultures were approximately 4.2mM. The sulphate concentration in the control batches was reduced from approximately 4.2mM to 0mM within 16 days.

The cultures treated with 1.85mM and 3.7mM *o*-cresol followed a similar trend as the control cultures, the sulphate concentration being greatly reduced by day 16 to 0.3mM and 1.1mM respectively. The greater the concentration of *o*-cresol, the slower the rate of sulphate reduction (Figure 3.1). Total elimination of sulphate from the cultures treated with 1.85mM and 3.7mM *o*-cresol was observed after 20 and 24 days respectively.

Sulphate concentrations in the batch cultures containing 4.6mM *o*-cresol initial decreased to approximately 3.1mM by day 11, the sulphate concentration then appeared to remain constant until day 22, where it then began to gradually decrease to 0mM by day 41.

Batch cultures contaminated with 5.6mM *o*-cresol showed no reduction in sulphate concentration until after day 30, at the end of the experiment the sulphate concentrations in these cultures had decreased to approximately 3.5mM. No reduction in sulphate concentration was noted throughout the

experiment for the batch cultures treated with *o*-cresol of 7.4mM, 9.3mM and 11mM.

In all of the batch cultures perturbed with *o*-cresol, an inhibitory effect on SRB was observed which increased with the concentration of *o*-cresol. At concentrations ≥ 7.4mM *o*-cresol complete inhibition of SRB was noted.

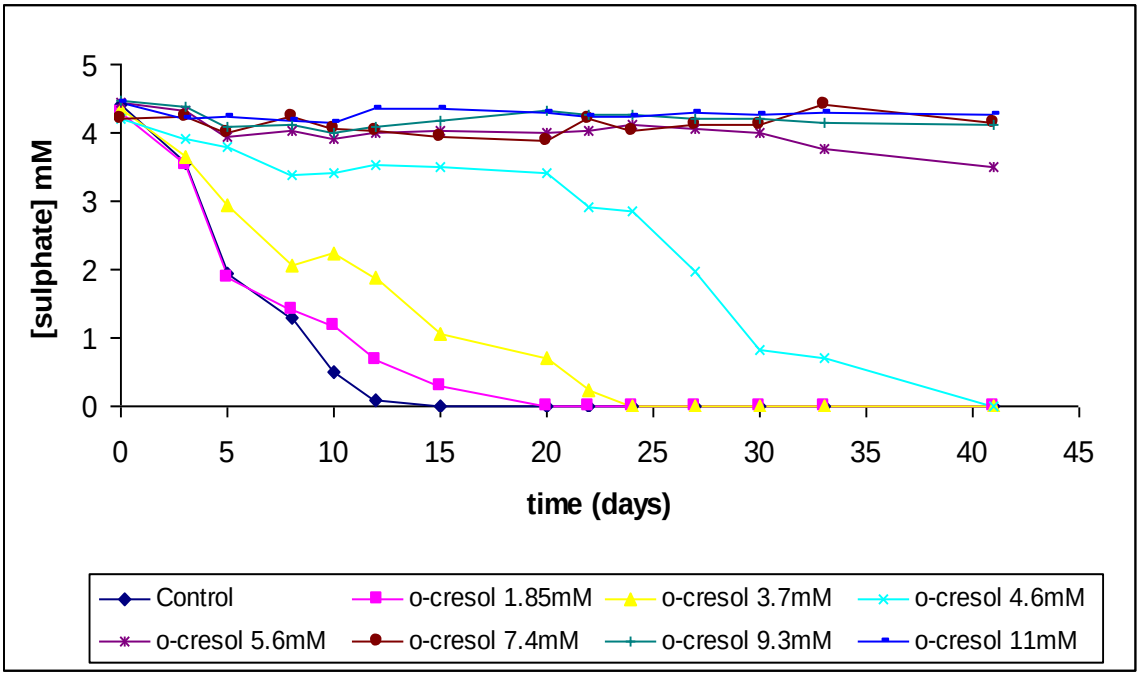


Figure 3.1 Sulphate reduction versus time for batch cultures containing o-cresol (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.2 pH *o*-cresol

The pH of the cultures was measured for the first 41 days of the experiment until the concentrations of VFA was no longer analysed. A previous scoping experiment to determine the concentration of DMP to be used in this study, indicated that the pH did not change enough to be monitored daily. The initial pH of the batch cultures appeared to have been affected slightly with

the addition of *o*-cresol, concentrations of ≥ 3.7mM *o*-cresol appear to increase the pH of the cultures slightly when compared to the control cultures (Figure 3.2). The initial pH of the batches was between 7.4 and 7.7. Another possible explanation for the variations in starting pH may be due to the varying concentrations of VFA in the inocula (Figure 3.3), the sludge was mixed well before addition to the batch bottles, and differences between pH were observed between the triplicates in each concentration set and the control set. In the control batch cultures and the cultures treated with 1.85mM *o*-cresol, the pH initially decreased to 6.6 by day 5 from 7.6 and 7.4 respectively. Subsequently the pH in the control cultures began to steadily increase to 8.0 by the end of the experiment, this increase in pH can be attributed to the breakdown and utilisation of VFA, when these acids are utilised, the pH will increase as a consequence of removal of acidic compounds from the medium. The pH of the cultures containing 3.7mM and 4.6mM *o*-cresol followed the same trend as the control cultures showing an initial decrease in pH to approximately 6.5 by day 5 then an increase to pH 8.0 by day 41; the initial increase in pH between days 5 and 15 appeared to be at a reduced rate when compared to the control cultures. After day 15 the pH values in both of the culture sets matched those of the controls. Batch cultures perturbed with 5.6mM *o*-cresol followed a similar trend in the pH profile as the controls e.g. falling to 6.6 by day 5, between days 5 and 15 a further drop in pH to 6.2 occurred at a slower rate. For the remainder of the experiment the pH in these cultures then increased to 7.6. The batch cultures which contained 7.4mM and 9.3mM *o*-cresol showed a preliminary decrease from pH 7.5 to 6.4. The pH of these cultures remained constant until day 15 where after a further decrease in pH values to 6.2 was noted by day 30. For the remainder of the experiment the pH in both cultures began to increase giving final

values of 7.0 and 6.7 respectively. In the batch cultures contaminated with 11mM *o*-cresol the pH decreased to 5.7 by day 5, by day 15 the pH had increased to approximately 6.6 where it remained until day 41.

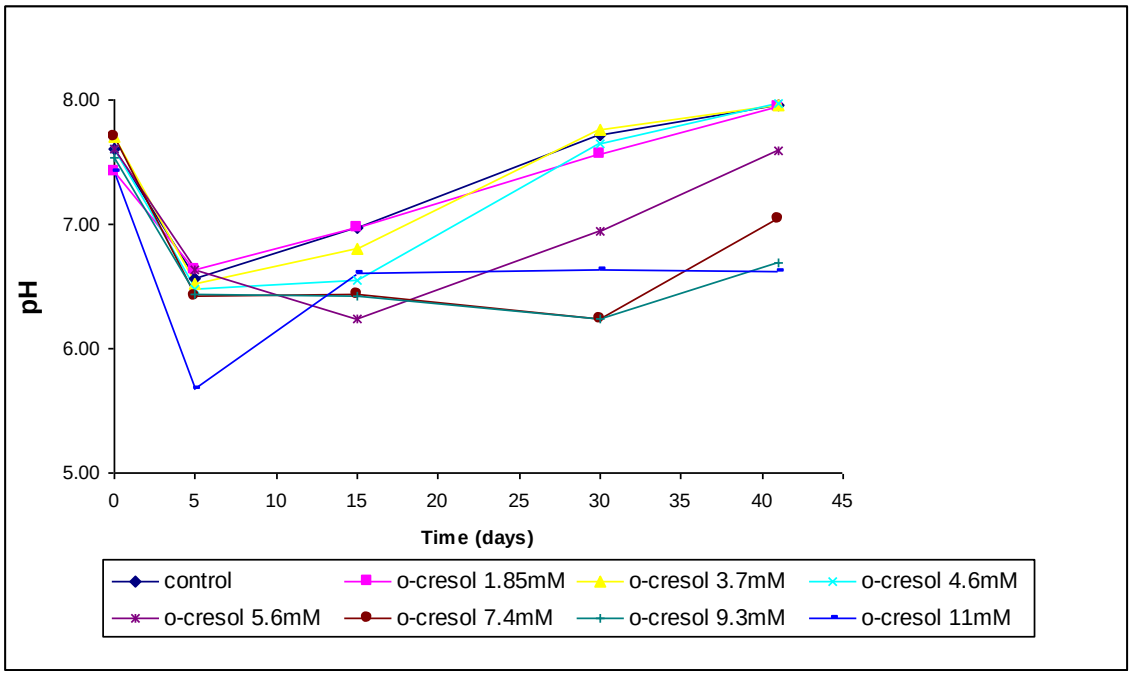


Figure 3.2 pH versus time for batch cultures containing o-cresol (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.3 Volatile fatty Acids *o*-cresol

The method employed for the analysis of all volatile fatty acids is detailed in the methods Section 2.2.4.

3.3.3.1. Acetate

The results obtained are shown in Figure 3.3. The key events in the process occur between 0-5 days (rapid increases in acetate was seen in all concentrations with the exception of 9.3 and 11mM) and then between 15 and 30 days where all cultures showed a significant decrease in acetate which is likely to have been reabsorbed by the cells, the exceptions were 9.3 and 11mM where acetate levels increased to 10mM and 8mM respectively by day 15 then remained constant for the remainder of the experiment

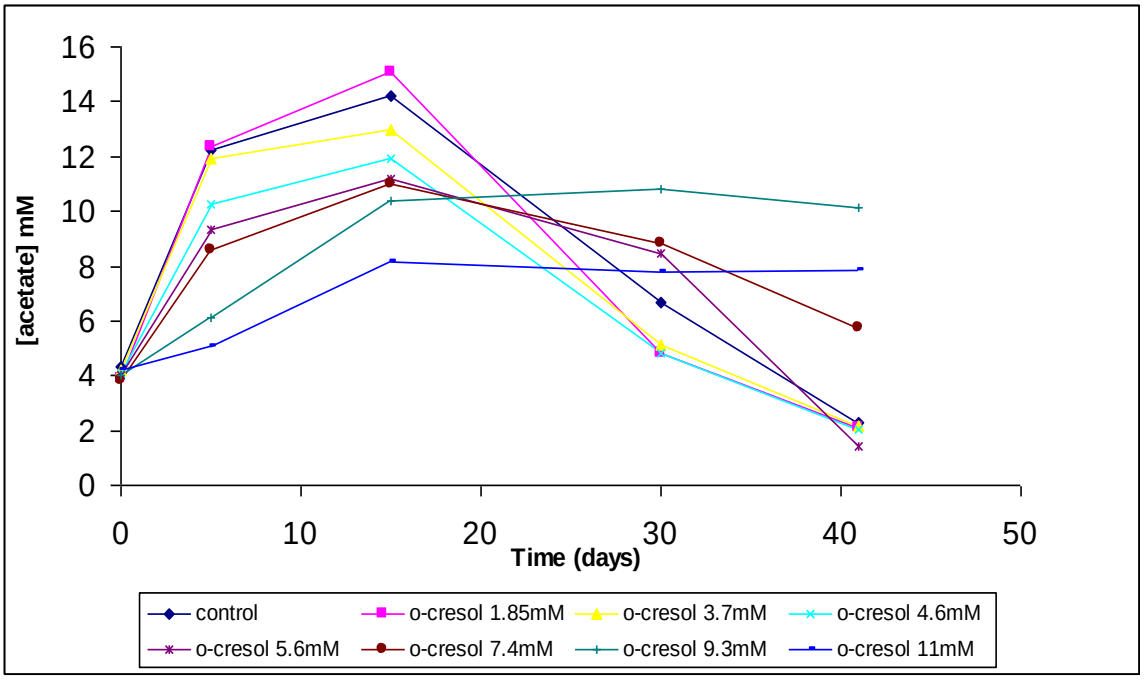


Figure 3.3 Acetate versus time for batch cultures containing *o*-cresol (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.3.2 Propionate

The results for the propionate concentrations of the batch cultures are shown in Figure 3.4. All cultures with the exception of those perturbed with 11mM *o*-cresol show a rapid increase in propionate concentration within the first 5 days. An increase is again observed in the cultures perturbed with concentrations of *o*-cresol ≥ 4.6mM from days 5 to 15, with the control cultures and those contaminated with the two lowest concentrations of *o*-cresol (1.85mM and 3.7mM) remaining almost constant during this period. After 15 days the propionate concentration decreases in cultures ≤5.6mM for the remainder of the experiment. The cultures containing 11mM o-cresol have a constant propionate concentration for the first 5 days then the concentration increases for the remainder of the experiment. Cultures containing 7.4mM and 9.3mM have a propionate concentration that appears to remain constant for the remainder of the experiment.

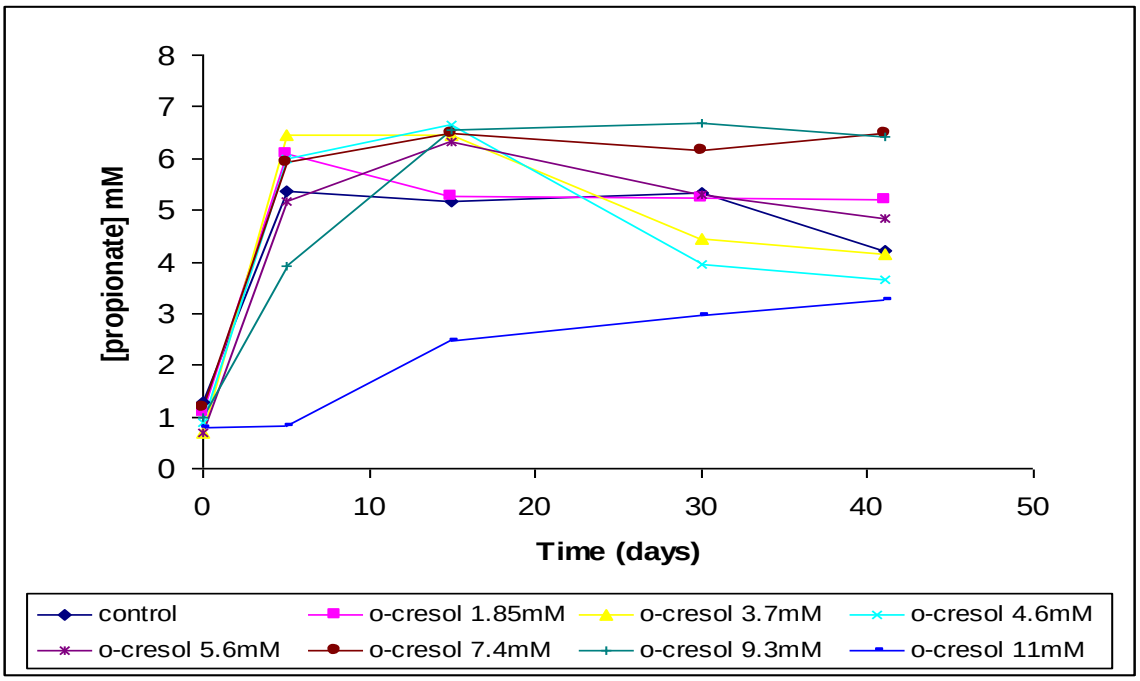


Figure 3.4 Propionate versus time for batch cultures containing *o*-cresol (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.3.3 Butyrate

The results obtained for butyrate concentrations are shown in Figure 3.5. The butyrate concentrations in the cultures show an increase within the first 5 days for all cultures, although it was observed that the concentration increased considerably slower in the cultures containing 11mM *o*-cresol. For the control cultures, and those perturbed with 1.85mM *o*-cresol, the butyrate levels fluctuate slightly between 5 and 30 days, but generally remained fairly constant. After 15 days all cultures started to show a decrease in butyrate concentrations with the exception of the cultures containing 9.3mM and 11mM *o*-cresol, which continued to increase to 3.3mM and 4mM respectively for the remainder of the experiment. The control cultures and those perturbed with 1.86mM and 3.7mM o-cresol were observed to have butyrate concentrations at the end of the experiment of 0.16mM, 01.6mM and 0.27mM respectively.

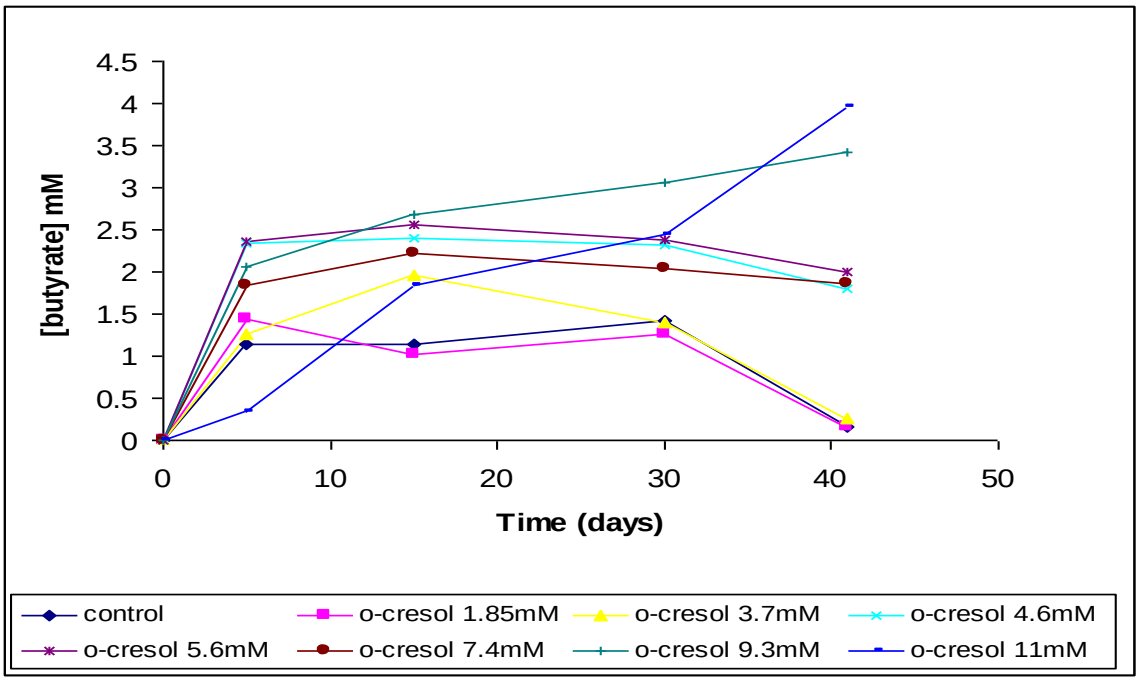


Figure 3.5 Butyrate versus time for batch cultures containing *o*-cresol (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.3.4 Iso-butyrate

The key results for iso-Butyrate concentrations can be seen in Figure 3.6. These were a rapid increase in iso-Butyrate concentrations in all cultures by day 5, with the exception of the two culture sets containing 9.3mM and 11mM (which had a slower increase in iso-butyrate). This increase continues in all cultures (except the cultures containing 1.85mM) until day 30, which is when the iso-Butyrate concentrations then begin to decrease. In the cultures containing the lowest amount of *o*-cresol (1.85mM), a slight decrease in concentration of iso-butyrate was observed between days 5 and 10 after this the concentration followed a similar trend to the control cultures with an increase, then a decrease after day 30. This decrease occurs in all cultures except those containing 9.3mM and 11mM *o*-cresol, which by the end of the experiment were still showing increasing levels of Iso-Butyrate (0.79mM and 0.65mM respectively).

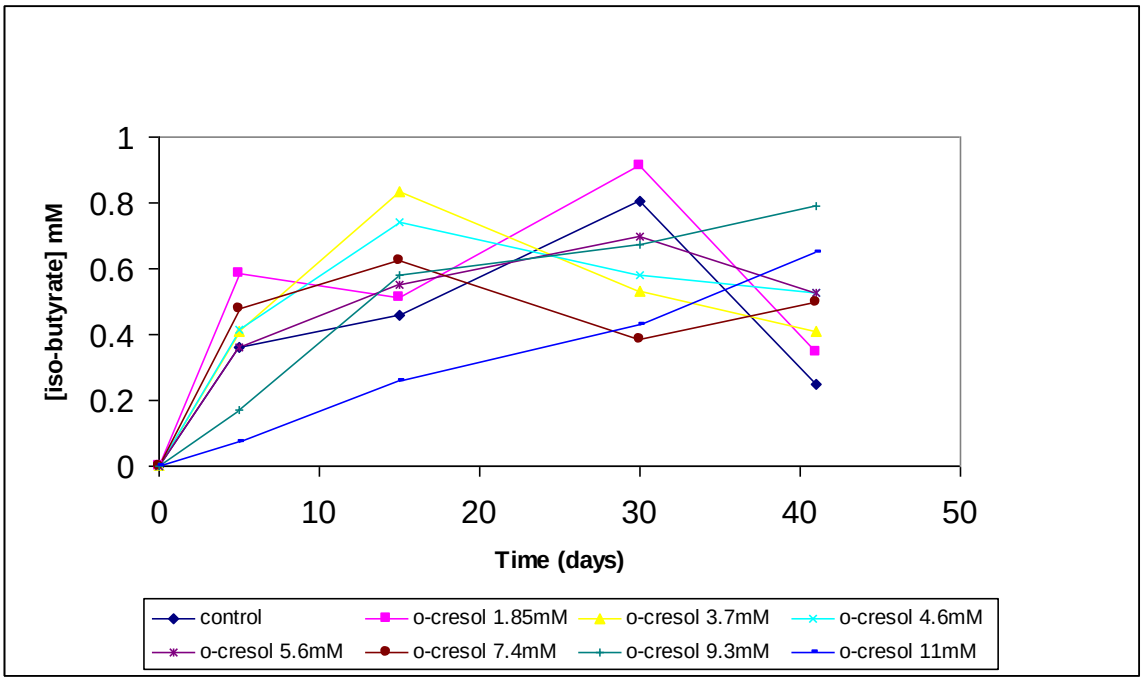


Figure 3.6 Iso-butyrate versus time for batch cultures containing *o*-cresol (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.3.5. Valerate

Analysis of the valerate concentration (Figure 3.7) showed that in all batch cultures the valerate concentrations increased from the onset of the experiment. A rapid increase was noted within the first 5 days in all cultures except those perturbed with the highest concentrations of *o*-cresol (9.3mM and 11mM). From day 5 to 10 all cultures continued to show an increase in valerate concentrations except for those contaminated with 1.85mM *o*-cresol, which showed a decrease. The control cultures and the cultures contaminated with 1.85mM *o*-cresol increase in valerate concentration from day 15 to 30 and the concentration rapidly decreases for the remainder of the experiment. The two culture sets contaminated with 9.3mM and 11mM *o*-cresol show an increase in valerate concentration for the entire experiment.

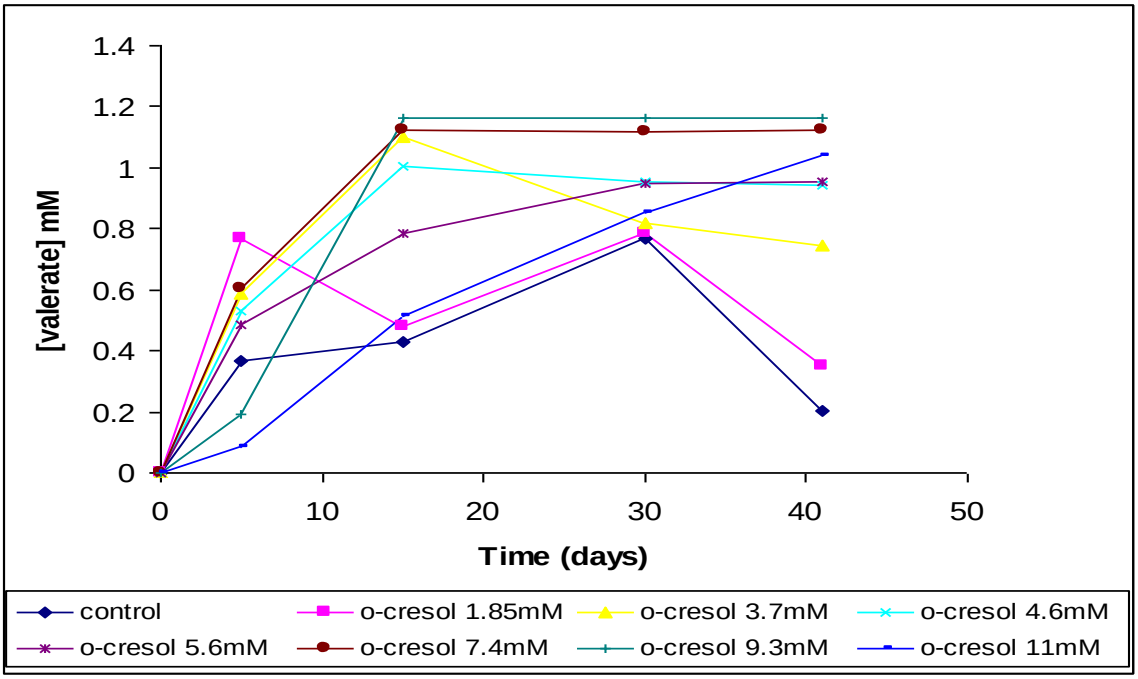


Figure 3.7 Valerate versus time for batch cultures containing *o*-cresol (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.3.6 Iso-valerate

The results for the iso-valerate concentrations in all of the batch cultures can be seen in Figure 3.8. The key events are a rapid increase in iso-valerate concentrations for the first 5 days in all cultures except those contaminated with the two highest concentrations of *o*-cresol (9.3mM and 11mM), which show an increase of iso-valerate at a slower rate. After day 30, a decrease in iso-valerate was observed in the control cultures (0% *o*-cresol), with all other cultures continuing to show an increase in iso-valerate.

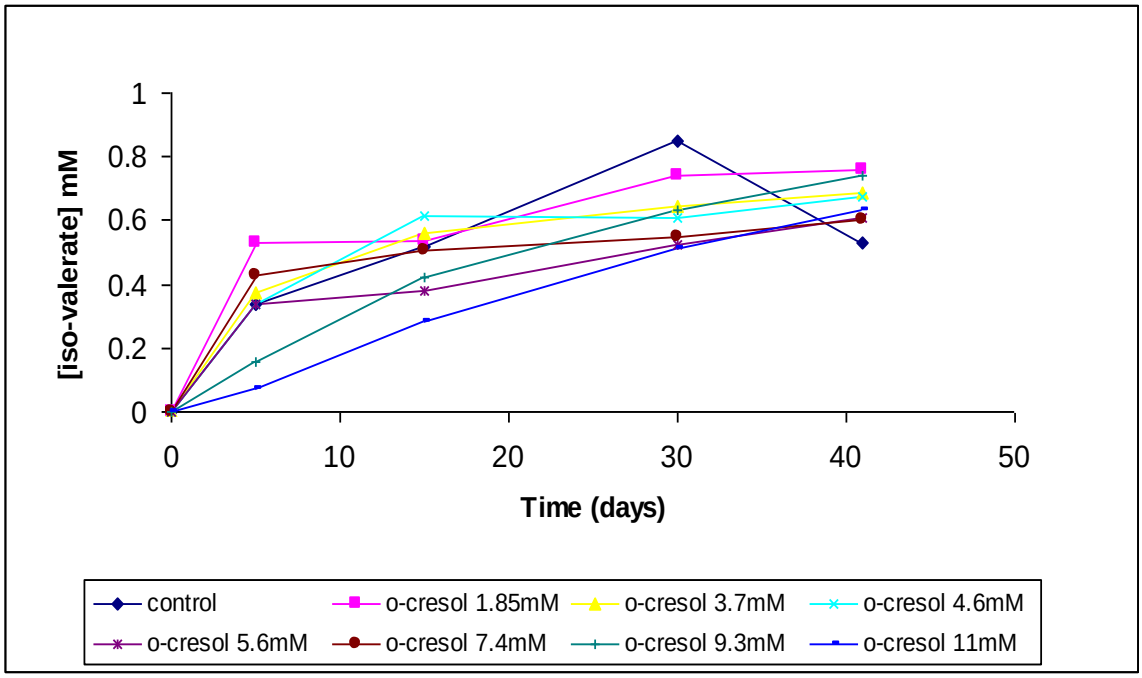


Figure 3.8 Valerate versus time for batch cultures containing *o*-cresol (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.3.7. Hexanoate

Figure 3.9 shows the hexanoate concentrations for the batch cultures. The concentration for hexanoate never went above 0.4mM in any of the cultures throughout the study. Even though the concentrations were low, some key results can be extracted from Figure 3.9, in all cultures there is an increase in hexanoate within the first 5 days. The hexanoate concentration in control cultures and those perturbed with 1.85mM *o*-cresol stays constant from days 5 to 10 then rapidly increases until day 30, this is followed by a sharp decrease in concentration for the remainder of the experiment. In all other cultures with the exception of those containing 9.3mM and 11mM *o*-cresol the concentration of hexanoate increased form the onset of the experiment until day 15 where it gradually decreased for the remainder of the experiment. The hexanoate concentrations in the cultures with the highest concentrations of *o*-cresol increased from the start of the experiment until the end.

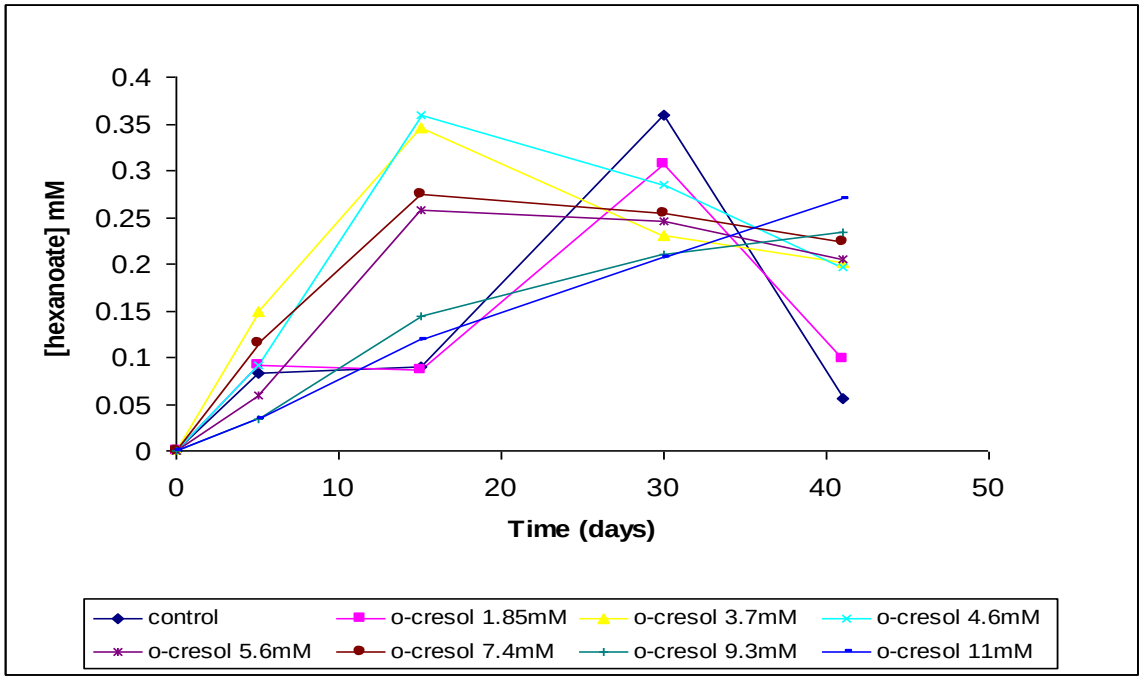


Figure 3.9 Hexanoate versus time for batch cultures containing o-cresol (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.4. Methane *o*-cresol

A GC technique was employed to measure the total amount of methane gas produced by the cultures (Section 2.2.3). Figure 3.10 shows the cumulative methane concentrations for the batch cultures. An initial lag period in methane production that lasted approximately 17 days was noted. The methane produced during this period was ≤ 1.72μmol/ml for all cultures and does not appear to be linked to the amount of *o*-cresol added. The methane production in the control cultures increased rapidly after the initial lag period giving a final total methane concentration of 21μmol/ml by day 47.

Total methane production in the batch cultures containing 1.85mM and 3.7mM *o*-cresol followed a similar pattern to the control batches, although at a slightly reduced rate, which appeared to be directly proportional to the amount of *o*-cresol contained in the cultures, giving final methane gas concentrations as 20.1μmol/ml and 17.5μmol/ml respectively.

The batch cultures treated with 5.6mM and 7.4mM *o*-cresol showed an increase in methane production, after the lag period, and by day 38 the total methane gas in these cultures was 11.3μmol/ml and 9.4μmol/ml respectively. Between days 38 and 47 the rate of methane production appeared greatly reduced giving final concentrations of 13.1μmol/ml and 10.5μmol/ml respectively. Total Methane gas production in the cultures perturbed with 7.4mM *o*-cresol after the lag period was reduced; reaching 2.6μmol/ml by day 31, after this point an increase in methane production was noted with the total methane produced by the end of the experiment being 9.39μmol/ml. The cultures contaminated with 9.3mM and 11mM *o*-cresol showed little change in methane concentration after the lag period until the end of the

experiment with final concentration of 3μmol/ml recorded in both sets of batch cultures.

All concentrations of *o*-cresol appear to have an inhibitory effect on methanogenisis, which is directly proportional to the *o*-cresol concentration, with almost complete inhibition being observed at *o*-cresol concentrations ≥ 9.3mM (figure 3.10).

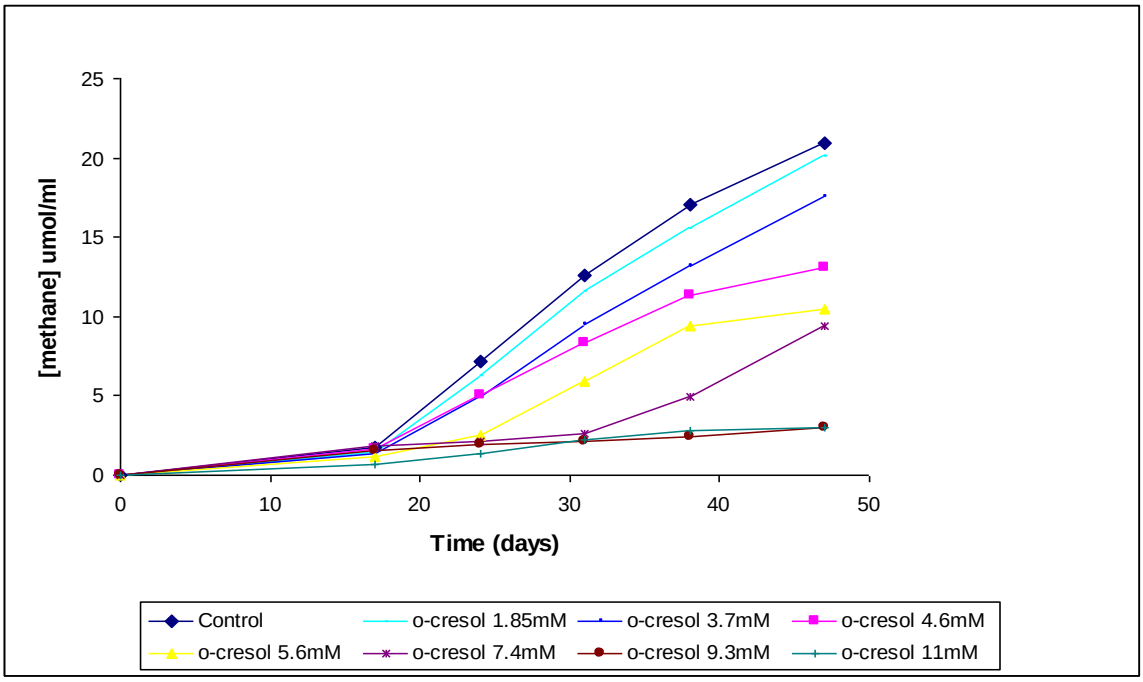


Figure 3.10. Methane production versus time for batch cultures containing *o*-cresol

(Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.5 Sulphate reduction 2,4-Dimethylphenol

An initial experiment on the effects of DMP on the anaerobic batch cultures was performed using lower DMP concentrations (0.08, 0.16, 0.33, 0.49, 0.62, 0.82 and 1.23mM); the sulphate reduction results from this experiment are presented in Appendix I (Figure I.1). From these results it was determined that the DMP concentrations had to be increased, to ensure complete inhibition at the higher levels of DMP.

The concentration of sulphate in batch cultures was measured by ion chromatography (Section 2.2.2). In all batches the initial sulphate concentration was approximately (4.0mM).

It can be seen from Figure 3.11 that, in the control batch culture, the concentration of sulphate was reduced from approximately 4.2mM to 0mM within 6 days. The concentration of sulphate in the batch cultures containing 0.41mM, 0.82mM, and 1.23mM DMP was also greatly reduced within 6 days, to 0.38mM, 0.64mM and 1.11mM respectively, however it can be seen that the sulphate reduction is faster at lower concentrations of DMP. The general trend of sulphate reduction in these cultures was similar to the control cultures with the total elimination of this compound occurring within 11, 22 and 33 days, respectively. The batch culture, which contained 1.64mM DMP, displayed a more gradual reduction in sulphate concentration, falling from 4.2mM to 0.94mM over the course of 41 days. When DMP was added to batch cultures at the higher concentration of 2.46mM, the sulphate concentration remained at 4.2mM until day 16, thereafter decreasing to 3.13mM and remaining at this concentration for the remainder of the experiment (41 days). In the batch cultures, which contained 3.28mM and 4.1mM, DMP the concentration of sulphate remained the same throughout the experiment (4.2mM). In all batch cultures supplemented with DMP there

was an inhibitory effect on the population of SRB, which increased with the concentration of DMP (Figure 3.11). At concentrations ≥ 3.28mM complete inhibition of SRB was noted.

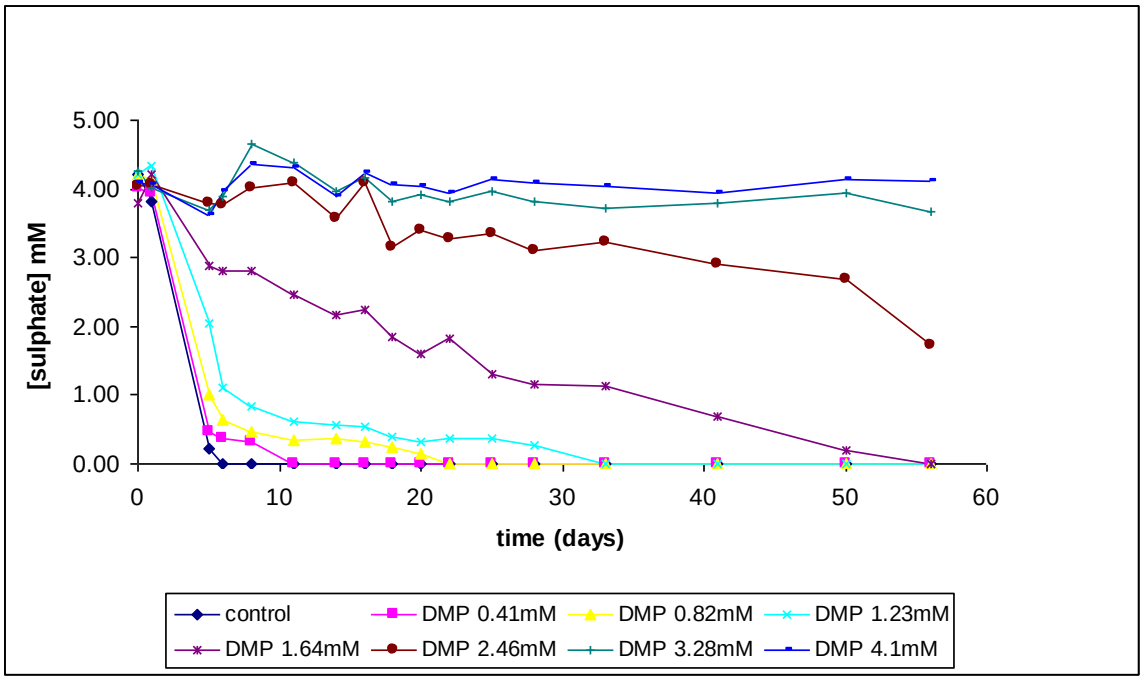


Figure 3.11 Sulphate reduction versus time for batch cultures containing 2, 4-dimethylphenol. (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.6 pH 2,4- Dimethylphenol

The pH of the batch cultures was measured at various intervals throughout the 41 days of the experiment (Section 2.2.1). The results show that the addition of DMP did not appear to alter the pH of the batch cultures on day 0 (Figure 3.12) unlike the addition of *o*-cresol, which appeared to be linked to pH (Section 3.3.2). The initial pH of the cultures was between 7.4 and 7.8,

these values were independent of DMP concentration. The variations in initial pH are explained in Section 3.3.2.

In the control cultures and the culture containing 0.41mM DMP, the pH decreased from 7.6 and 7.4 respectively, to 7 in the first 6 days of the experiment. After this point the pH gradually increased to 8. The pH of the batch cultures treated with 0.82mM and 1.23mM followed a similar trend to that of the control cultures, displaying an initial decrease in pH from 7.4 to 6.7 after 6 days, then a gradual increase to pH 7.8 at day 41. The batch cultures that contained concentrations of DMP of 1.64mM, 2.46mM and 3.28mM all showed a preliminary decrease from pH 7.8, 7.5 and 7.7 respectively to 6.5. The pH by day 6 in all of these cultures remained at this concentration until day 28. Between days 28 and 33 a slight increase in pH was noted in these cultures and the pH further increased to 7 by day 41. In the batch cultures supplemented with the highest concentration of DMP, 4.1mM, the pH value decreased from approximately 7.6 to 6.5 by day 6, after which the pH value increased slightly at day 16. The pH then dropped to 6 at day 28 and then increased to pH 6.7 by the end of the experiment. DMP concentrations ranging from 0.82mM to 4.1mM all appeared to influence the pH of the batch cultures over time when compared to the control cultures. At the highest concentration 4.1mM DMP the pH does not return to its initial value, this may indicate an inhibitory effect on the bacteria that utilise VFA, which in turn, keeps the pH concentrations lower than those in the control cultures.

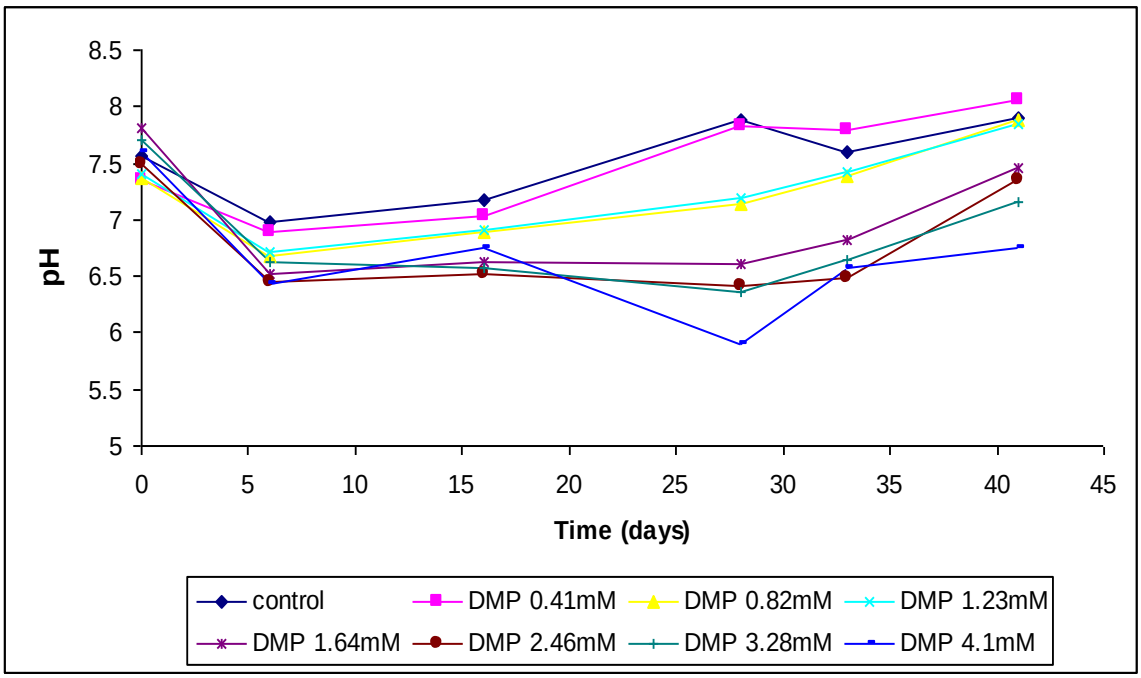


Figure 3.12 pH of batch cultures containing varying concentrations of 2, 4-dimethylphenol. (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.7 Volatile Fatty Acids (VFA's) 2,4- Dimethylphenol

VFA production in the batch cultures was measured by GC (Section 2.2.4), this method allowed for the analyses of seven VFA's: acetate, propionate, iso-butyrate, butyrate, valerate, iso-valerate and hexanoate. In the case of all VFA's studied, an initial difference in acid concentration was observed, the differences in initial concentration might be explained by the inocula containing different ratios of bacterial populations

3.3.7.1 Acetate

The results can be seen in Figure 3.13. The key events are an initial rapid increase in acetate concentrations in all batch cultures from day 0 to day 6. After day 6 all cultures with the exception of those perturbed with the highest concentrations of DMP (2.6mM, 3.28mM and 4.1mM) showed a decrease in acetate concentration for the remainder of the experiment. The cultures containing 2.6mM of DMP showed an increase in acetate until day 28, then decreased rapidly for the remainder of the experiment. After day 6 the concentrations of acetate in the two culture sets contaminated with the highest concentrations of DMP (3.28mM and 4.1mM) remained constant for the remainder of the experiment.

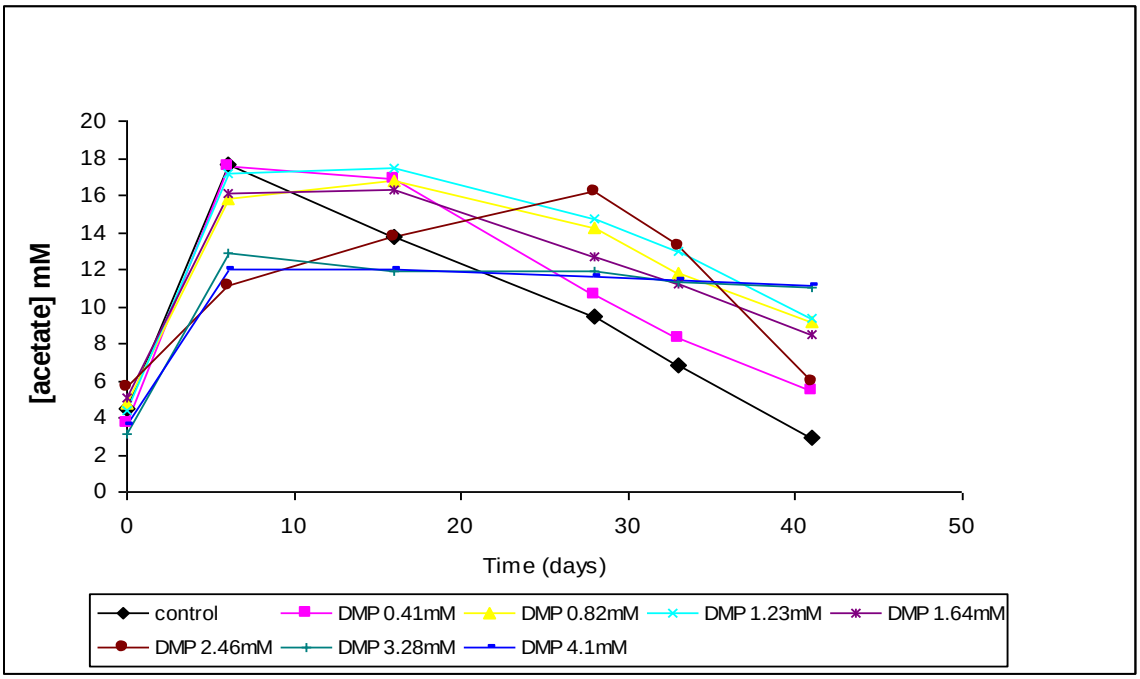


Figure 3.13 Acetate concentration versus time for batch cultures treated with DMP. (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.7.2 Propionate

Propionate results from the cultures can be seen in Figure 3.14. In the first 6 days, all cultures showed an increase in propionate concentration, this increase appears to be linked to the DMP concentrations with the concentration of propionate decreasing with the concentrations of DMP increasing. After Day 6 the propionate concentrations in all cultures do not fluctuate significantly with the exception of the cultures perturbed 2.46mM, where a slight decrease in propionate after day 28 then had an increase after day 33 was observed.

The key observations were the initial increase in propionate then the concentration remaining constant.

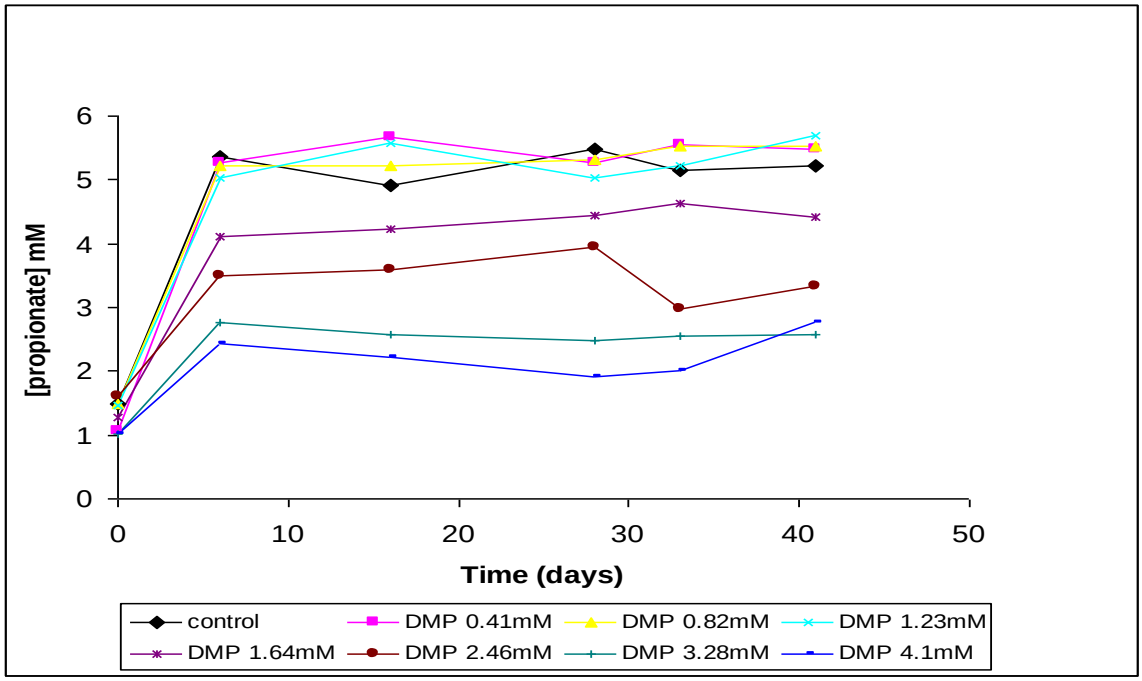


Figure 3.14 Propionate concentration versus time for batch cultures treated with DMP. (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.7.3 Butyrate

Butyrate concentrations can be seen on Figure 3.15. The key observations made were, all cultures showed an increase in butyrate concentration until day 6. The butyrate concentrations of the two culture sets perturbed with 3.28mM and 4.1mM were significantly higher after 6 days than any of the other batch cultures, this appears to be due to an inhibition of butyrate utilisation. After day 6 the butyrate concentrations appears to remain relatively constant until day 28. After day 28 the only notable decrease in butyrate concentrations occur in the control cultures and those containing 0.41mM DMP.

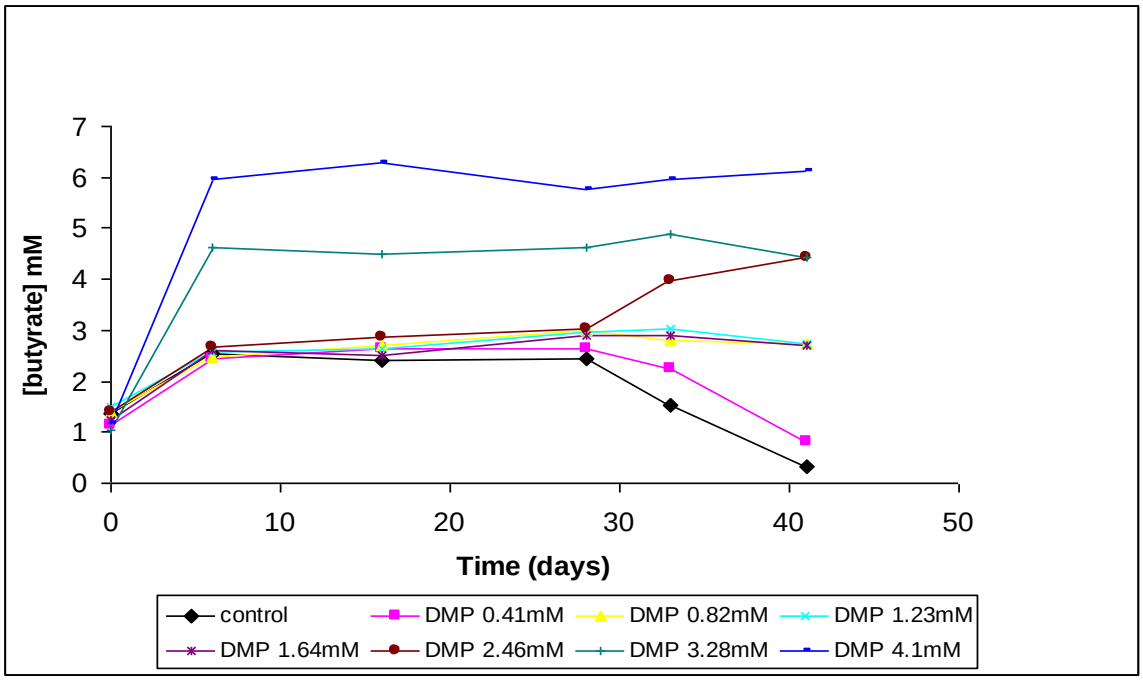


Figure 3.15 Butyrate concentration versus time for batch cultures treated with DMP. (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.7.4 Iso-butyrate

Iso-Butyrate levels in the batch cultures can be observed in Figure 3.16. The concentrations of iso-butyrate in all cultures are low throughout the experiment. All cultures show an initial increase in iso-butyrate concentrations. The notable results are that there appears to be a significant decrease in iso-butyrate concentrations after day 28 only in the control cultures.

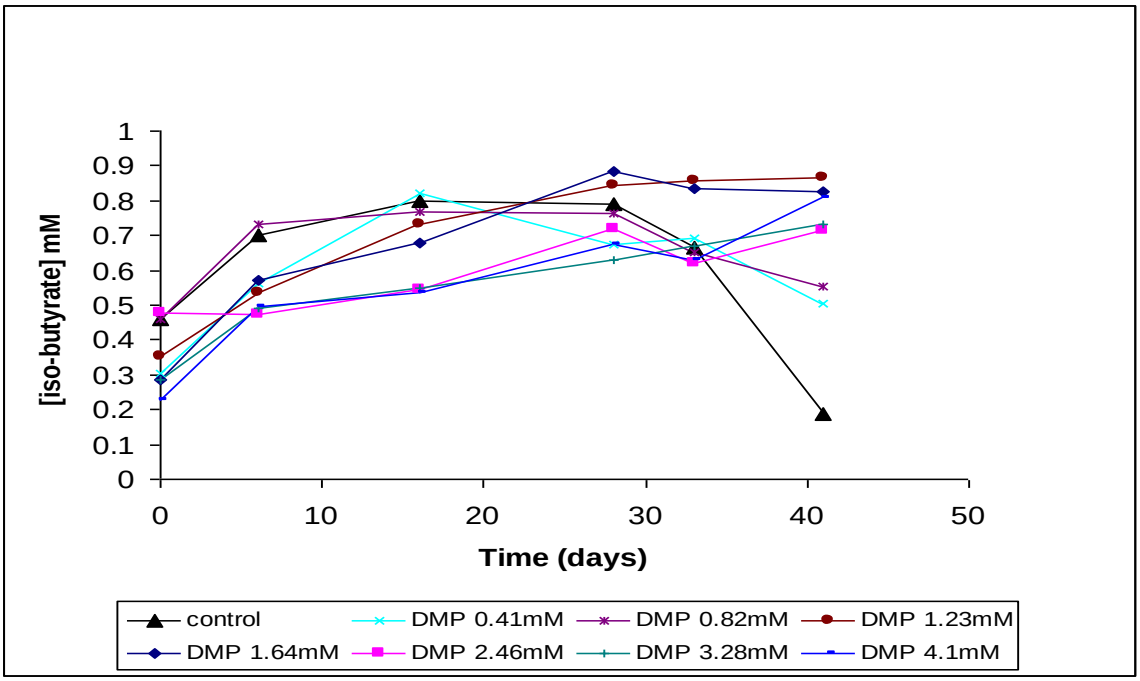


Figure 3.16 iso-butyrate concentration versus time for batch cultures treated with DMP. (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.7.5 Hexanoate

Hexanoate concentrations observed in this experiment can be seen in Figure 3.17. Cultures containing DMP concentrations ≤ 1.23mM showed slight fluctuations in hexanoate concentrations throughout the experiment with the levels of hexanoate being below 0.4mM throughout. The cultures containing DMP at concentrations of 1.64mM, 2.46mM, 3.28mM and 4.1mM showed an increase in hexanoate throughout the experiment with a hexanoate concentration of 1.2mM being recorded in the culture containing 4.1mM DMP by the end of the experiment.

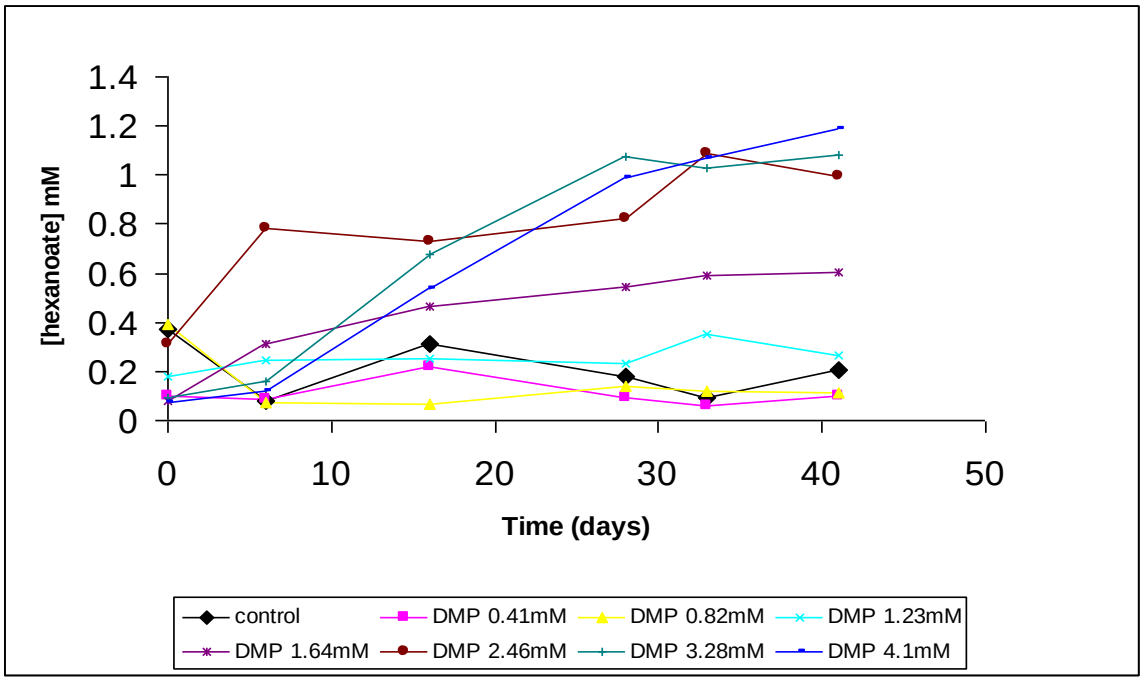


Figure 3.17 Hexanoate concentration versus time for batch cultures treated with DMP. (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.7.6 Valerate

Valerate concentrations from the batch culture experiment can be seen in Figure 3.18. The concentrations of valerate in the control cultures and those containing DMP concentrations of 0.41mM, 0.82mM and 1.23mM remain below 1mM throughout the experiment. The cultures perturbed with the higher concentrations of DMP (1.64mM to 4.1mM) show a rapid increase in valerate in the first 6 days, which appears to be linked to DMP concentrations (the higher the DMP concentration the higher the valerate concentration), the valerate in theses cultures then decreases until day 33 where it then remains constant for the remainder of the experiment. The cultures contaminated with the highest concentration of DMP (4.1mM) show an initial increase in valerate concentrations of approximately 5mM within the first 6 days this is double any other valerate increase noted.

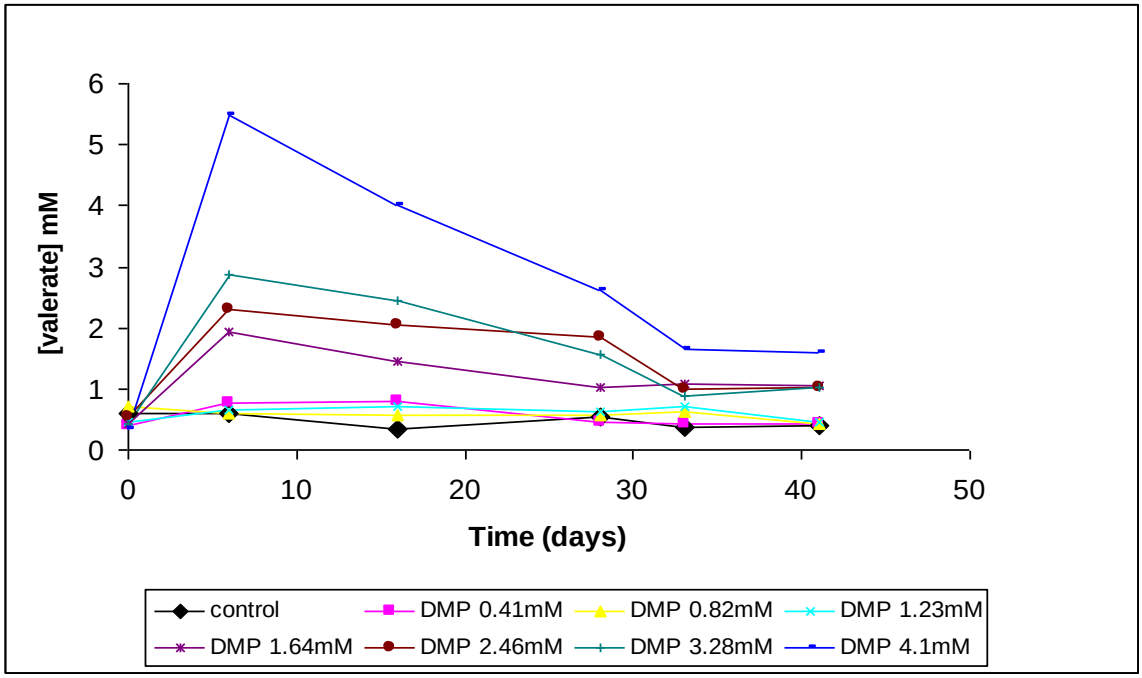


Figure 3.18 Valerate concentration versus time for batch cultures treated with DMP

(Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.7.7 Iso-valerate

The concentrations for iso-valerate in all the control cultures and the cultures perturbed with DMP increased for each culture (Figure 3.19). The amount of DMP added did not appear to have any effect on iso-valerate concentrations, which increased for every culture giving values ranging from 0.76mM to 1.1mM, these concentrations do not appear to be linked to the amount of DMP present in the cultures.

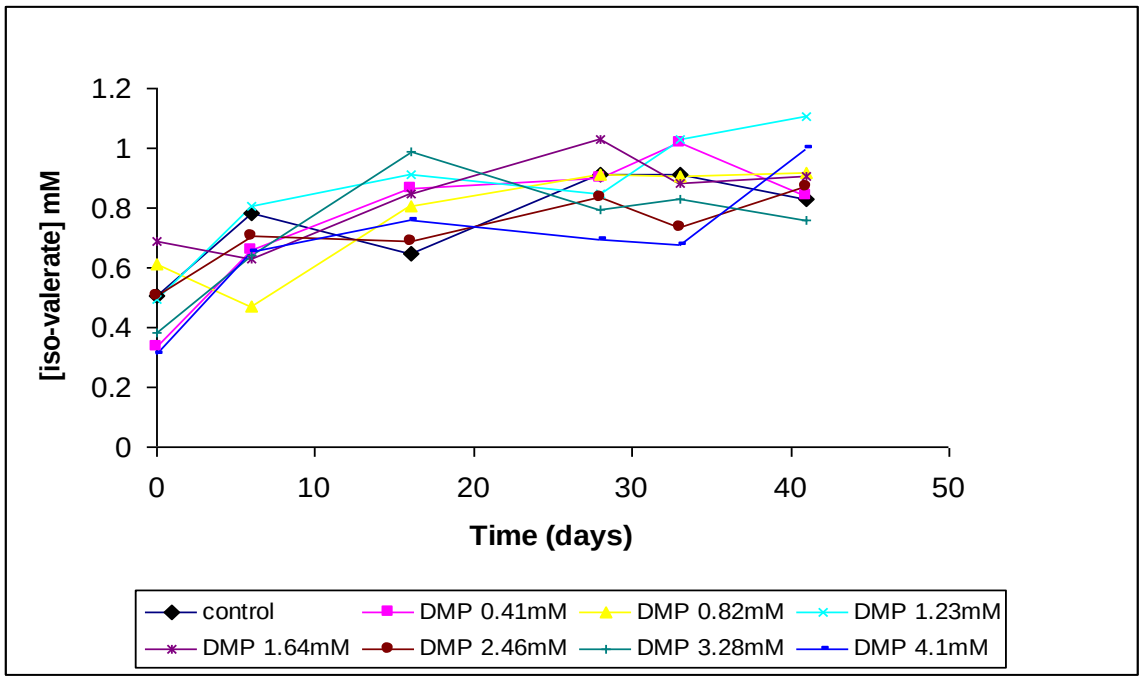


Figure 3.19 iso-valerate concentration versus time for batch cultures treated with DMP (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.3.8 Methane 2,4-Dimethylphenol

The total methane gas produced by the batch cultures was measured by gas chromatography (Section 2.2.3). An initial lag period in the production of methane was observed in the control culture and all cultures treated with DMP (Figure 4.11). This period lasted approximately 15 days and the methane produced was below 2μmol/ml for all cultures. In the control culture methane production started to increase with time after the lag period and reached a final concentration of 23μmol/ml by the end of the experiment. The total methane gas production in the 0.41mM DMP cultures was 17.8μmol/ml by day 41. Methane gas production in the batch cultures perturbed by: 0.82mM, 1.64mM, 2.46mM and 3.28mM, increased after the lag period reaching concentrations between 7.2μmol/ml and 8.9μmol/ml after 41 days. By day 15, batch cultures treated with 4.1mM produced slightly more methane than all other cultures. By the end of the experiment the total amount of methane produced by these cultures reached 5μmol/ml.

All the batch cultures treated with DMP produced lower concentrations of methane when compared to the control cultures. These results indicate that methane-producing bacteria may have been inhibited, and this effect increases with increasing concentrations of DMP. In the batch cultures contaminated with 4.1mM total methane production reaches approximately 1/5 of that for the control cultures.

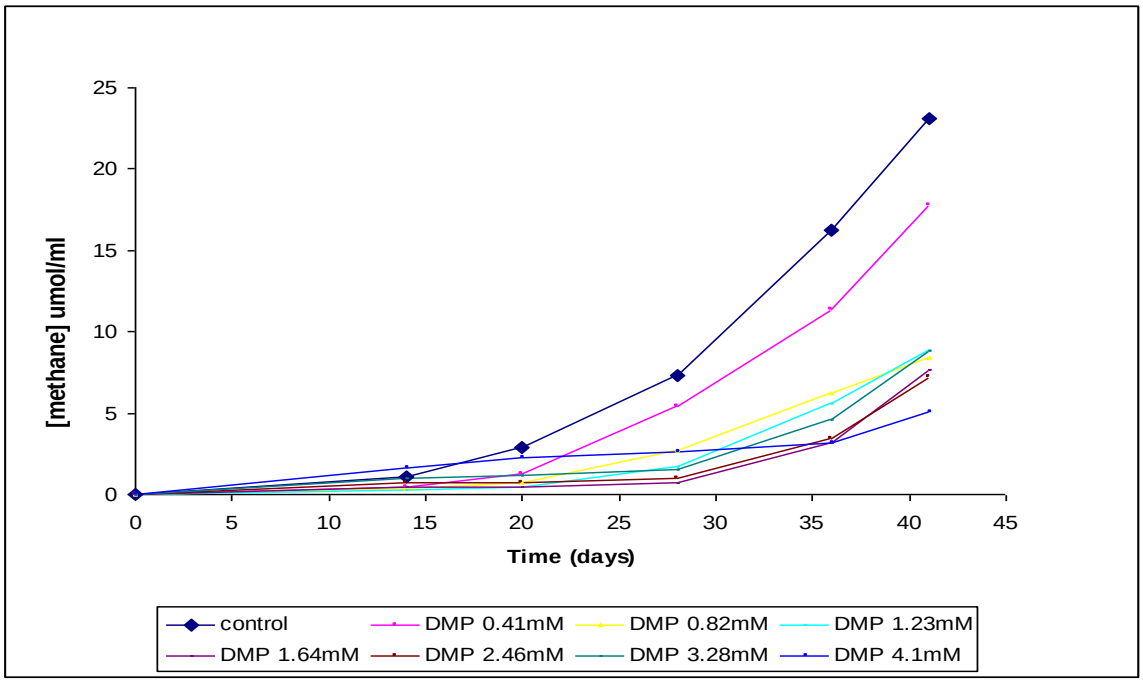


Figure 3.20 Methane gas production versus time for batch cultures containing DMP (Standard deviations can be found in Appendix I, the standard deviations are acceptable)

3.4 Discussion of batch culture results

3.4.1 Sulphate

In the batch culture experiment investigating the effects of *o*-cresol, the range of concentrations of *o*-cresol chosen were selected on the basis that there would be no inhibition (0% *o*-cresol) through to total inhibition (11mM *o*-cresol – based on the work in the PhD thesis by Sulisti (1994)). It was shown that even the lowest concentration of *o*-cresol, 1.85mM exhibited an inhibitory effect on the reduction of sulphate. This reduced rate in sulphate reduction is possibly caused by the inhibition of sulphate reducing bacteria. As the concentration of *o*-cresol increases, the rate at which sulphate is reduced decreases. At *o*-cresol concentrations of ≥ 4.6mM there appears to be a lag period in which the rate of sulphate reduction is greatly diminished, the concentration of sulphate decreases less than 1mM within the first 20 days, after this the rate of sulphate reduction increases and by day 40 no sulphate is recorded. The lag period may be due to the inhibition of fermentative bacteria in the cultures, which reduce the cellobiose to smaller sugars, and VFA, which are the substrates for SRB (Section 3.3.3). Another possibility is that the concentration of *o*-cresol is sufficient to slow the growth of the SRB enough to reduce their efficiency, or that the lag period is due to the bacteria acclimatising to the *o*-cresol, thereafter reducing sulphate as normal. An even greater lag period was noted in the cultures containing 5.6mM *o*-cresol.

Concentrations of *o*-cresol ≥ 7.4mM completely inhibit sulphate reduction and appear to have a toxic effect on the bacteria, in hindsight the bacteria may have needed even longer to acclimatise and may have been an extended lag period which is still a toxic effect, the experiment was stopped after 40

days due to a time restraint on the study. It may have been possible to extend the incubation period of the batches and this may be future work.

A previous study by Holmes *et al.,* (2002) showed the inhibitory effect of *o*-cresol on anaerobic bacteria using refuse as an inocula. With the supplementation of 3.5mM sulphate, *o*-cresol concentrations of 4mM and 6mM were shown to inhibit sulphate reduction by 4.5% and 19.4% respectively. Return activated sludge was used as the inocula in the present study, and the amount of sulphate added was 4.2mM. Analysis of the results of the cultures perturbed with 3.7mM and 5.6mM showed an inhibition of sulphate reduction of 33% and 91% respectively. The difference in percentage inhibition of sulphate reduction between these two studies could be due to the microbial activity of the inocula, with refuse being more active than sludge, and the differing bacterial populations of the two inocula; also the number of bacteria present in the different inocula could be a factor, with the refuse having a higher number of bacteria. The nature of the inocula meant that standardisation was very difficult as the populations and the relevant concentrations of the different populations could impact on the results obtained. In order to try to decrease the variability in the inocula, for each set of experiments the inocula came from the same batch of sludge.

As with *o*-cresol (Section 3.3.1) increasing the concentration of the 2,4-dimethylphenol decreased the amount of sulphate reduced. All concentrations of DMP utilised exhibited an inhibitory effect on sulphate reduction, even at the lowest concentration studied (0.41mM) inhibition occurred albeit slight. All concentrations of DMP appeared to have an inhibitory effect on the bacterial consortium and possibly the sulphate reducing bacteria, as the concentration increased the inhibitory effect

increased and less sulphate was reduced, thus indicating a toxic effect on the consortium.

At concentrations of DMP ≥ 3.28mM complete inhibition of sulphate reduction was observed, indicating that these concentrations of DMP are extremely toxic to the bacterial consortium. In the *o*-cresol batch culture study, sulphate reduction was completely inhibited by *o*-cresol concentrations ≥ 7.4mM; this is double the concentration of DMP needed to produce the same effect.

3.4.2 pH

The pH of both culture sets observed had similar trends. The control cultures pH followed the typical trend for anaerobic microbial associations with pH dropping initially during the acidogenic stage, then rising with the utilisation of VFA. According to Hilkiah Igoni *et al.,* (2008), the bacteria involved in anaerobic digestion have a pH range between 6 and 8, with 7 being optimum. This is in agreement with the results found herein, where the pH of all cultures, except the one supplemented with the highest value of *o*-cresol, remained within this range. In both batch culture experiments (*o*-cresol and DMP) it can be hypothesised that the initial decrease in pH was due to the breakdown of the long carbon chain cellobiose into shorter chain VFA's. Following the decrease in pH, a steady increase in pH was observed for the remainder of the experiment. This is due to the utilisation of the VFA's by SRB, acetogens and methanogenic bacteria.

At the lowest concentration of *o*-cresol, 1.85mM no effect on the pH of the culture was observed (Figure 3.2). In the cultures containing the higher concentrations of *o*-cresol, the pH decreased and stayed at this level for some time before gradually increasing. This is due to the inhibitory effect that *o*-

cresol exhibits on the acidogenic bacteria, which produce VFA's and those that utilise them. Figures 3.3 to 3. 9 show inhibition of VFA utilisation at high concentrations of *o*-cresol, which left higher concentration of the acids therefore keeping the pH lower. At the highest concentration of *o*-cresol, the pH dropped below 6 in the first 5 days, then increased slightly and stayed constant until the end of the experiment. The substantial decrease in pH is due to the fermentative breakdown of cellobiose to VFA's and the apparent lack of utilisation of these acids showing almost complete inhibition of the acetogenic and other VFA utilising bacteria, such as methanogenic and sulphate reducing bacteria. These results are in agreement with those of Sulisti (1994), which showed that at higher concentrations of *o*-cresol, the pH remained low due to the build up of volatile fatty acids. Thus it can be seen that in experiments with refuse (Sulisti, 1994) and sludge (the present study) the inhibition by o- cresol resulted in a similar change in pH.

In the batch cultures perturbed with DMP, the pH of the cultures followed the trend observed for *o*-cresol, with the control cultures and those perturbed with the lowest concentration of DMP having similar pH at all time points. The pH remained lower than the controls in cultures containing DMP at concentrations ≥ 1.23mM. This reduction in pH followed by the slow increase back to a neutral pH was due to the increase of VFA in the cultures indicating that DMP exhibited an inhibitory effect on the acetogenic bacteria, which utilised these compounds, the slow increase of pH maybe due to bacterial acclimatisation to DMP allowing utilisation of the VFA. In Section 3.3.5 it was shown that the highest concentrations of DMP completely inhibited sulphate reduction. This may be due to sulphate reducing bacteria to be inhibited. This bacterial group can utilise acetate as an electron donor; therefore, an indication of their inhibition may be no decrease in the acetate

concentration after 5 days coupled with the lack of sulphate reduction. This is in direct contrast with the control culture and those with DMP concentrations ≤ 1.64mM. The culture containing 1.64mM DMP does not show as much pH recovery as the cultures containing less DMP even though it shows a decrease in acetate this is due to the accumulation of other VFA's (Section 3.4.3).

3.4.3 VFA

The volatile fatty acids studied in this experiment are shown in the Table 3.1. The breakdown of long carbon chain sugars (substrates) to VFA in anaerobic consortia has been well documented (Aboutboul *et al.,* 1995; Buyukkamaci and Filibeli, 2004; Demirel and Scherer 2008). VFA's are directly linked to the pH of the cultures. With the formation of these acids the pH decreases, as the acids are utilised the pH increases.

Table 3.1 Table of the VFA studied in this experiment

Name	Chemical Formula
Acetate	CH_3COOH
Propionate	CH_3CH_2COOH
Butyrate	$CH_3 (CH_2)_2COOH$
Iso-butyrate	$CH_3 (CH_2)_2COOH$
Valerate	$CH_3 (CH_2)_3COOH$
Iso-valerate	$CH_3 (CH_2)_3COOH$
Hexanoate	$CH_3 (CH_2)_4COOH$

Long chain carbon sources are broken down to acetate and the other VFA's (Watson-Craik *et al*., 1991; Molino *et al*., 2013). The VFA's with 3 or more carbons are further broken down to acetate, hydrogen and bicarbonate by acetogenic bacteria (Hansen *et al*., 2001; Kotelnikova, 2002). Acetate is then utilised by acetoclastic methanogenic bacteria and the hydrogenotrophic methanogens utilise bicarbonate and hydrogen to produce methane (Christensen *et al*., 2000; Hansen *et al*., 2001; Kotelnikova, 2002). Acetate is the major substrate for the production of methane in the environment. It is estimated that two thirds of methane produced is due to acetate utilisation (Taconi *et al*., 2007). Acetate, propionate and butyrate can all be utilised by sulphate reducing bacteria as electron donors (Cao *et al*., 2012).

As might be expected with cellobiose (a carbohydrate) as the main substrate (Section 3.1), acetate, propionate and butyrate were the most abundant acids possibly due to them being the shortest chain acids, therefore products of the breakdown of the larger chain acids e.g. hexanoate, and due to the breakdown of cellobiose to glucose, which can be further broken down to

acetate and butyrate. Valerate, iso-valerate, iso-butyrate and hexanoate were formed at low concentrations. This observation agrees with previous studies (Sulisti, 1994; Nitayapat, 2003; Buyukkamaci and Filibeli, 2004) although these studies did use different substrates.

The effect of *o*-cresol on the production and utilisation of VFA's can be clearly seen in the results (Figures 3.3–3.9). In the case of acetate, the control cultures and those perturbed with 1.85mM *o*-cresol produced more acetate by day 15 than any other cultures. As the concentration of *o*-cresol increased the amount of acetate produced decreased during the early stages of the experiment. This indicates the possible inhibition of the acidogenic bacteria and the acetate producing acetogens. After day 15 all cultures containing *o*-cresol concentrations ≤ 7.4mM showed a decrease in acetate concentration, as the acetate began to be utilised by the methanogenic bacteria (Section 3.3.4); the rate of utilisation reduced as the *o*-cresol concentration in the cultures increased. Acetate was directly utilised by the acetoclastic methanogens and Sulphate reducing bacteria, therefore the decrease in acetate utilisation may be an indicator that *o*-cresol appeared to inhibit these bacteria (Jones and Simon, 1985; Liamleam and Annachhatre, 2007). Cultures containing concentrations of *o*-cresol ≥ 9.3mM showed a reduced production rate for acetate and after day 15 the acetate concentration in these cultures remained constant. These results show that the concentrations used in the current study have a major inhibitory effect on acetate production and appear to inhibit its utilisation completely.

The utilisation of propionate and butyrate is not normally energetically favourable and they both have positive red-ox potentials; See Figure 3.21

Reaction	ΔG°′ per reaction (kJ)
$CH_3CH_2COO^- + 3H_2O \leftrightarrow CH_3COO^- + H^+ + 3H_2 + HCO_3^-$	+76.5
$CH_3(CH_2)_2COO^- + H_2O \leftrightarrow 2CH_3COO^- + H^+ + 2H_2$	+48.1

Figure 3.21 Gibbs free energy equations for the conversion of propionate and butyrate. Adapted from Voolapalli and Stuckey (1999).

Butyrate will be degraded before propionate due to its more negative ΔG°′. Factors that affect the degradation of these compounds are the amount of acetate present and the hydrogen partial pressure, as well as redox conditions needing to be energetically favourable. McInerney and Byrant, (1981) stated that the hydrogen partial pressures have to be less than 2×10^{-3} atm and 9×10^{-5} atm to make it possible for the utilisation of butyrate and propionate respectively. The problem in the degradation of these compounds is that their products are the very compounds that inhibit their degradation of acetate and hydrogen, and therefore the organisms that utilise propionate and butyrate need to form a synotrophic relationship with hydrogen scavenging bacteria, either sulphate reducing bacteria (SRB) or methanogenic bacteria (MB). Voolapalli and Stuckey (1999) and Schmidt and Ahring (1993) showed that the degradation of both propionate and butyrate can be enhanced with the utilisation of hydrogen scavenging bacteria and that the rate of VFA degradation is linked to H_2- consuming activity.

In the control cultures of the o-cresol batches a slight decrease in both propionate and butyrate was noted after day 5, possibly due to sulphate reducing bacterial activity creating more favourable conditions for the utilisation of these acids coupled, with the use of hydrogen decreasing its

partial pressure. When the sulphate was completely reduced, it appears that these acids were no longer utilised indicating that the SRB may utilise them. After day 30 the concentrations began to fall due to the lowering of the acetate and hydrogen concentrations by the methanogens. The results from the cultures containing *o*-cresol ≥ 1.85mM and ≤ 5.6mM show that at the lower concentrations of *o*-cresol, the initial concentrations of propionate are higher than those of the controls. This could be due to slight inhibition of sulphate reducing bacteria inhibiting the utilisation of propionate. In the case of butyrate the cultures containing *o*-cresol except for the highest concentration, had higher concentration of butyrate than the controls; this again is due to the increase in hydrogen partial pressure due to possible inhibition of sulphate reduction causing a decrease in butyrate utilisation. During methanogenesis the propionate concentrations in these cultures appears to decrease at a faster rate than in the control cultures and this can be linked to the lower acetate concentrations in these cultures, which in turn caused less inhibition. Butyrate mineralization rates during methanogenesis are slower in the batch cultures containing *o*-cresol compared to the control cultures, showing an inhibitory effect on the bacteria that utilise butyrate. At the highest concentrations of *o*-cresol, the propionate and butyrate concentrations increased slowly from the onset of the experiment and never decreased due to the inhibition of acetogenic bacteria, SRB and MB. The accumulation of these acids can have an inhibitory effect on all the bacteria in the consortia, whether from the compound toxicity (Mara and Horan, 2003) or the lowering of pH to concentrations that are inhibitory to the bacteria. When the pH is lowered, propionate exhibits a more toxic effect on bacteria since it exists in an un-dissociated HPr form, this un-dissociated form has

greater membrane permeability than the propionate ion which results in a greater toxic effect (Mara and Horan, 2003).

The remainder of the VFA's produced were at concentrations below 1.5mM and while the results show differences in concentration between the utilisation and production of these acids, the concentrations are low enough not to contribute greatly to the overall systems. The results from these acids although found in low concentrations, do show that when compared to the control cultures, the concentration of *o*-cresol in the batches affects the production and utilisation of these acids. On analysis of the results it appears that as with propionate and butyrate, these acids are affected by H_2 partial pressure and degradation is only seen in low concentrations of *o*-cresol after the onset of sulphate reduction and methanogenisis.

The results obtained from the control cultures of o-cresol contamination (Section 3.3.3) showed the typical trend for VFA production and utilisation during anaerobic digestion. The control culture in the DMP study followed a similar trend with the exception of propionate, which showed no decrease in concentration.

All concentration of DMP added to the cultures affected the acetate concentrations. In the control cultures, acetate was rapidly produced within the first 6 days and then decreased for the remainder of the experiment, as it became an electron donor for firstly SRB, and then secondly acetoclastic methanogens. The initial acetate concentration in the cultures appeared to decrease with the increase of DMP, indicating a possible toxic effect on the acidogenic bacteria and the acetate producing acetoclastic bacteria. Analysis of other VFA concentrations showed an increase in larger VFA

concentrations with the exception of propionate, as the concentration of DMP increases indicating the inhibition of acetogenic bacteria.

By day 16 the acetate concentrations in cultures containing DMP ≤ 1.64mM began to fall, this coincided with the beginning of methanogenesis (Figure 3.20). The rate at which the acetate was utilised after this point was reduced when compared to the control, indicating that there was a decrease in methanogenic activity possibly due to the inhibition of acetogenic methanogens. It could not be determined whether the hydrogenotrophic methanogens were inhibited by these concentrations of DMP. At DMP concentrations greater than 0.41mM the methane concentration in these batch cultures did not rise above 10μmol/mL compared to the control and 0.41mM DMP cultures reaching concentrations of 23μmol/mL and 17.8μmol/mL respectively, indicating that DMP at these concentrations exhibited a significant toxic effect on methane production possibly due to inhibition of the acetoclastic methanogens.

In this set of batch cultures all the DMP contaminated cultures and the control cultures showed an initial increase in propionate concentration, which appeared to be dependent on DMP concentration, with the increase in DMP concentration less propionate was produced. After the initial increase, the propionate concentrations in all cultures remained constant indicating that the acid was not being utilised, or being utilised and produced at the same rate. In the control culture, the propionate concentration showed slight fluctuations indicating utilisation and production, but on the whole the concentration remained constant indicating that it had not become energetically favourable to breakdown propionate. Propionate breakdown was observed in the control of the *o*-cresol batch cultures, the fact that it did not appear to occur here could be due to seasonal changes in the bacterial

inocula, even though the sewage sludge was taken from the same treatment plant, there can be a seasonal shift in the bacterial populations for example between the cold winter and the warm summer e.g. Desunes *et al.,* (2007) showed, seasonal changes in nitrate reducing bacteria. As parameters change, for example temperature, shifts in bacterial populations are to be expected.

The butyrate concentrations of all the cultures, including the control culture, showed an initial increase with the breakdown of longer chain carbon substrates. Analysis of the results indicates that the greater the concentration of DMP, the greater the initial concentration of butyrate observed. The concentration of butyrate remained constant for the rest of the batches with the exceptions of the control culture and those contaminated with 0.41mM DMP. At the highest concentration of DMP, butyrate concentrations were 6mM. From the results it appears that at a concentration of DMP ≤ 2.46mM, initial butyrate concentrations were lower than the batches with higher DMP concentrations, this could be due to butyrate being utilised during sulphate reduction. On analysis of the iso-butyrate chart (Figure 3.16) it can be seen that its concentration increases slightly, Wang *et al.,* (1999) showed that during the breakdown of butyrate, iso-butyrate can be formed along with propionate due to decarboxylation. In a study by Sulisti (1994) butyrate utilisation was observed to have followed the β-oxidation pathway and produced 2 molecules of acetate, the inocula used in this study was, however, refuse. It appears from the literature that the specific degradation pathway is dependent on the presence of particular bacterial species rather than bacterial groups, therefore different anaerobic consortia would utilise butyrate in different ways.

The results show that even at the highest concentrations of DMP the bacteria that produce VFA during the acidogenic stage appear not to be inhibited by DMP. At the highest concentrations of DMP the butyrate concentrations did not decrease indicating the possible inhibition of acetogenic bacteria and also the possibility that, due to inhibition of other bacterial species e.g. SRB and methanogenic bacteria, the hydrogen partial pressure remains too high for it to be energetically favourable to breakdown this compound.

Valerate concentrations in the cultures increased with the concentration of DMP ≥ 1.64mM and showed an initial increase within 5days. The greatest increase was noted in the cultures contaminated with 4.1mM DMP. The valerate concentrations in all of these cultures then decreased until day 33 then remained constant for the remainder of the experiment. In the control cultures and those contaminated with DMP ≤ 1.32mM, the valerate concentrations remained below 1mM throughout the experiment. These results show that concentrations of DMP ≥ 1.23mM appeared to have an inhibitory effect on the utilisation of valerate as mentioned before. This could be due to the inhibition of the SRB, which in turn increased the hydrogen partial pressure; the valerate concentrations in these cultures then decreased which could be due to the onset of methanogenisis. Even though only low concentrations of methane are produced this could be enough to lower the hydrogen partial pressure indicating that the methane produced may be due to hydrogenotrophic methanogens, rather than acetoclastic methanogens which appear to be inhibited due to the lack of acetate utilisation (Figures 3.13 and 3.20).

Iso-butyrate and iso-valerate were only produced in small amounts for all of the cultures. Their production can be linked to the acidogenic stage in which VFA's are produced. These two acids were also produced in small amounts

during the breakdown of their isomers, butyrate and valerate, the small amounts of these acids produced during this anaerobic digestion can be compared to the amounts detected during the *o*-cresol batch study and the study by Sulisti (1994).

Hexanoate concentrations in the cultures remained low during the course of the experiment. The concentrations in the control culture and cultures contaminated with DMP ≤ 1.23mM remained relatively constant and below 0.4mM. During the experiment cultures contaminated with DMP ≥ 1.64mM showed an increase in hexanoate concentrations due to the inhibitory effect these concentrations of DMP exhibit on the acetogenic bacteria which utilise hexanoate.

It can be clearly seen that all concentrations of DMP effect the VFA concentrations in these cultures. At the highest concentrations, inhibitory effects were noted on the acetate producing acetogens and the initial concentrations of VFA in these cultures indicate an inhibitory effect on the fermentative bacteria, which produce VFA. In the cultures contaminated with lower concentrations of DMP, the VFA concentrations were affected by the inhibition of the hydrogen scavenging SRB and methanogens, which caused an increase in hydrogen partial pressure as acetate was formed inhibiting the breakdown of larger VFA.

3.4.3 Methane

Methane is the end product for the complete mineralization of organic carbon by anaerobic digestion. There are two distinct groups of

methanogenic bacteria acetoclastic and hydrogenotrophic. The acetoclastic (heterotrophic) methanogens utilise acetate to produce methane and the hydrogenotrophic (autotrophic) use hydrogen and carbon dioxide (Kotelnikova 2002; Hansen *et al*., 2001; Christensen *et al.*, 2000). It is estimated that two thirds of the methane produced in nature is from acetate, the remaining third is made from hydrogen and carbon dioxide (Ferry 1992). The two equations for the production of methane are shown in Figure 3.22.

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \qquad (1)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad (2)$$

Figure 3.22 Methane production by acetoclastic methanogens (1) Ferry (1992), and hydrogenotrophic methanogens (2) Schink (1997)

In the *o*-cresol batch study, methanogenisis did not occur until after 17 days, in all cultures including the control. Other authors have also noted this lag period before methanogenesis (Wang *et al.,* 1984; Sulisti, 1994; Holmes *et al.,* 2002). It is due to the time taken for the slow growing methanogenic community to establish, this may be due to the redox conditions not being reduced enough for methanogens. The methanogens need the other bacterial groups to breakdown the larger carbon sources into substrate they can use and reduce the conditions within the cultures. Another possible reason for the lag period is that in the early stages of the batch cultures there is activity in sulphate reduction and it has been previously noted that sulphate reducing bacteria can outcompete methanogens for H_2 (Abram and Nedwell, 1978; Kristjansson *et al* 1982; O'Flaherty *et al.,* 1998)

O-cresol appears to have an inhibitory effect on methane production at all concentrations. At the lowest concentration of *o*-cresol (1.85mM) there is a

slight decrease in methane production when compared to the control cultures. At this concentration of *o*-cresol, acetate concentrations are comparable with the control cultures indicating its production and use are similar, therefore it is the possible that the reduction in methane is due to inhibition of the hydrogenotrophic methanogens. Holmes *et al*., (2002) noted that hydrogenotrophic methanogens appear to be more susceptible to *o*-cresol than acetoclastic methanogens. As the amount of *o*-cresol increased the amount of methane produced decreased. Cultures containing ≤ 7.4mM *o*-cresol showed a possible inhibition of hydrogenotrophic methanogens and a possible inhibition of acetoclastic methanogens, the acetate concentration in these cultures is lower than the control cultures due to inhibition of other bacterial groups, therefore there is less substrate for the methanogens hence less methane. At *o*-cresol concentrations ≥ 9.3mM methane production appears to be completely inhibited, in these cultures acetate concentrations are lower than the other cultures, but they slowly increased to usable concentrations. The lack of acetate utilisation points towards the inhibition of the acetoclastic methanogens, this may be directly from the toxic effect of *o*-cresol or due to the inhibition of other bacterial groups within the consortium.

The methane concentration in the DMP batch control cultures is comparable with the results for the control cultures in the *o*-cresol batch experiment, although the lag period for methanogenisis differs between both experiments, possibly due to differences in the activity of the inocula. The control cultures in both experiments followed a similar trend and produced ≥ 20mM of methane over the course of the experiment. On analysis of the control culture results in both experiments, an initial lag period was

observed in both. DMP appeared to have a much greater inhibitory effect on methane production than *o*-cresol. The batch cultures from the previous chapter contaminated with 3.7mM o-cresol produce approximately the same concentration of methane as the cultures from this experiment contaminated with 0.41mM DMP. At all other concentrations of DMP the total methane produced was below 10μmol/mL, although by the end of the experiment the methane production rates appeared to be increasing. From the results it cannot be determined whether the hydrogenotrophic methanogens were inhibited. In the *o*-cresol batch experiment the cultures contaminated with the lowest amount of *o*-cresol 1.64mM produced and utilised the same amount of acetate, therefore it was reasoned that the drop in methane production in these cultures, compared to the control, was due to the inhibition of hydrogenotrophic methanogens rather than acetoclastic methanogens, which was backed up by a study conducted by Holmes *et al*., (2002). In this experiment the acetate concentrations were different in the controls and all cultures contaminated with DMP, therefore the reduction in methane production could be linked to the inhibition of acetoclastic methanogens. From the results obtained it is unclear if the hydrogenotrophic bacteria are inhibited.

3.5 Summary

In conclusion to this chapter it can be seen form the results of both sets of batch cultures that *o*-cresol and 2,4-dimethylphenol have an inhibitory effect on the bacterial consortia.

The results show that DMP has a greater effect on the bacterial consortia than *o*-cresol does. Kahru *et al*., (2000) showed that cresols were more toxic than phenols and that xylenols (including DMP) were more toxic than cresols (Table 1.3). Holmes *et al.,* (2002) found that o-cresol is 3-10 times as effective

as a bacteriocide than phenol. It is believed that with the addition of a methyl group, the phenolic compound becomes more toxic, but its solubility is reduced. It has been previously reported that xylenols are more toxic than cresols that are in turn more toxic than phenol (Acuña-Argüelles et *al.*, 2003). A study conducted by Kahru *et al.*, (2000) showed that the cresol isomer p-cresol was more toxic than phenol and 2,4-dimethylphenol in some tests (Table 1.3). The difference in toxicity results in previous studies could be due to the toxicity testing methods as mentioned in the previous chapter's summary. The difference between 2,4-dimethylphenol and *o*-cresol is an additional methyl group at carbon 2 on the aromatic ring. The additional methyl group is thought to be responsible for the decrease in solubility of DMP's when compared to cresols (see Table 1.2). Analysis of results for both the o-cresol and the DMP experiments show, inhibition of normal anaerobic metabolism, with the increase in concentration of both these compounds.

As both compounds are polar narcotics then there toxicity increases with their hydrophobicity and hydrogen bond activity (Veith and Broderius, 1990). Due to DMP being less soluble than *o*-cresol it has an increased hydrophobicity, this coupled with the extra hydrogen bond indicates that DMP will be more toxic than o-cresol and backs up the results in this chapter. Analysis of the data produced, by this experiment showed that the sulphate reduction was more susceptible to both contaminants than methane production and acetogenic processes. Holmes *et al.*, (2002) observed that sulphate reducing bacteria were more susceptible to *o*-cresol than methanogens. Although it must be noted; in the present study the *o*-cresol and DMP were added to the batch cultures on day 1, therefore the sulphate reducing bacteria might not have had enough time to establish, making them more susceptible to the phenolic compounds. Another reason that sulphate

reduction appears to be affected more is that the population sizes of the different bacterial groups may be different, there may not be as many sulphate reducing bacteria, therefore a greater toxic effect would be observed.

The batch cultures allowed the analysis of an anaerobic bacterial community as a whole and enabled the toxicity testing of *o*-cresol and DMP against various groups of anaerobic bacteria found in the environment. The results from this study provide a good indication of the toxic concentrations of *o*-cresol and DMP, but do not show the exact effect it exhibits on the specific bacterial groups.

The batch cultures showed inhibition of bacterial processes by *o*-cresol and DMP e.g. methanogenisis and sulphate reduction. Although it was unable to show if the effect on these processes was directly or indirectly due to the presence of *o*-cresol and DMP. The anaerobic organisms in these batch cultures have synotrophic relationships with each other (Section 1.3), therefore the inhibition of one group of bacteria can have a knock on effect on other bacteria within the consortium (Parkes and Senior 1988). For example if the sulphate reducing bacteria are inhibited, then sulphate may not be reduced leading to an inhibitory effect on other bacterial species and the breakdown of long chain carbon substrates may be greatly diminished, causing the starvation of other species that utilise the shorter carbon chain products as well as not allowing for the development of reduced redox conditions which are important for methanogenisis. Luostarinen, (2005), stated that sulphate can have an inhibitory effect on other bacterial groups in a bacterial consortium, for example methanogens. SRB also act as a hydrogen sink, if they are inhibited the hydrogen partial pressure will increase causing

problems for the acetogenic phase, since these types of organisms have a synotrophic relationship (Vooplapalli and Stuckey, 1999).

The use of pure cultures would have enabled a more detailed analysis on the direct effects of the phenolic compounds on the individual bacteria found in anaerobic consortia. The reason pure cultures were not used was that in anaerobic growth consortia the bacterial groups have synergistic relationships with each other (Section 1.3). Therefore due to these relationships a concentration of *o*-cresol or DMP may affect the bacteria in a consortium more than if it was pure cultured, due to an indirect effect, caused by inhibition of other synotrophic groups within the consortium that produce important pre-cursors and create redox conditions needed by for the specific bacteria. It may also have been found with the use of pure cultures the bacteria in the consortia were offered more protection from DMP and *o*-cresol, due to their relationships with the other bacterial groups. Thus the concentration of *o*-cresol or DMP needed to produce a toxic inhibitory effect would be different when testing on pure cultures or bacterial consortia. Pure cultures would allow for a more detailed analysis of the toxic effects, but in anoxic environments the bacteria are present as consortia.

To enable further analysis of the effects of *o*-cresol and DMP on the individual bacterial groups continuous flow multi-stage systems are used (Chapter 4). These systems segregate the bacterial groups to enable a more detailed study of the direct effects of o-cresol and DMP on specific bacterial groups.

Chapter 4

Alkylphenol Toxicity using Continuous Flow Three-stage systems

4.1 Introduction

The studies conducted in Chapters 3, where batch cultures were employed, showed that *o*-cresol and 2,4-dimethylphenol exhibited inhibitory effects on the microbial populations of the cultures.

In both batch cultures, the inhibition of sulphate reduction by both *o*-cresol and DMP was observed, and the inhibition of methanogenic, acetogenic and acidogenic processes was also noted. However it was not possible, from these studies, to determine the effects on specific metabolic groups, for example the inhibition of methanogenisis was observed with the addition of each compound, however it was not possible to tell which methanogenic group, acetoclastic or hydrogenotrophic, was more susceptible to the compound. Although the results from the batch cultures showed inhibition of bacterial processes, it was unclear which bacterial groups were actually affected by *o*-cresol and DMP directly. Or whether due to the synergistic relationship of the batch cultures the processes were inhibited due to lack of substrates or possibly redox conditions being unfavourable.

In order to study the effects of *o*-cresol and DMP in more detail, multi-stage continuous flow systems were set-up. Multi-stage systems have been utilised in previous studies to enable the analysis of microbial interspecies interactions from samples isolated from landfill sites (James *et al*., 1998; Holmes *et al*., 2002).

In this study, 3-stage systems (Figure 1.4) were employed to examine the effects of the two compounds *o*-cresol and DMP on separated physiological groups of microorganisms. The experimental design of the systems allowed for the separation of the bacterial groups into distinct micro-niches, which occupied the different vessels of the systems depending on the spatial separation of terminal electron acceptors. This was achieved by adjusting

the media dilution rates using a Watson-Marlow peristaltic pump (Cornwall, U.K.), and also due to the media composition, which in turn enabled the formation of distinct bacterial habitat domains of specific physiological groups, while still allowing the overlap of activity domains which allowed interaction and communication between the different species (Holmes *et al*., 2002).

4.2 Experimental set up

4.2.1. Three-stage continuous culture system

Four multi-stage continuous flow systems were set up as shown in Figure 1.4, systems A, B, C and D (system C and D were used in the molecular section of this chapter). The system model was adapted from Holmes *et al*., (2002). It can be seen that the vessel sizes increased down the array, the volumes of the vessels were 330 mL for A1, B1, C1 and D1, 730mL for A2, B2, C2 and D2, and 1145mL for A3, B3, C3 and D3. Systems C and D were set up to investigate the combined toxicity of *o*-cresol and DMP (Chapter 5), although they were used in the molecular section of this chapter. In the systems, each vessel was modified using a three-way stopcock to allow the input of *o*-cresol and/or 2, 4-DMP. Aluminium foil covered each of the vessels to exclude light and a water thermo-circulator (U5-CTCV, Churchill Instrument Co., Uxbridge) was employed to keep the vessels at 30°C by pumping water through the vessels outer water jacket. The top vessels of each system were inoculated with 125ml from an active control batch culture which contained Cellobiose- VFA carbohydrate catabolising microbial associations, isolated from return activated sewage sludge, and allowed to stabilise for 7days. Anoxic conditions were maintained by continuous over

gassing of all vessels with OFN and after an initial stabilisation period the flow rate of the system was maintained at 14mlh^{-1}, regular pump tubing changes and pump checks ensured this. Gas traps were added to each vessel to prevent build up of hydrogen sulphide and carbon dioxide, the traps contained barium hydroxide (18gl^{-1}) and zinc acetate (1% w/v). The continuous gassing of the system vessels also ensured that the poisonous hydrogen sulphide (a product of sulphate reduction) did not inhibit any bacterial processes as it was continually flushed from the vessels by the OFN.

The growth medium supplemented with sulphate (4.2mM) and cellobiose (5.02mM) was then pumped in to the top vessels, using a Watson-Marlow peristaltic pump (Cornwall, U.K.) at an initial flow rate of 5mlh^{-1}. Once the top vessels became full, the next vessels in the array would begin to fill due to overflow from the top vessel. This enabled the spatial separation of the key bacterial groups.

Due to the increase in vessel size down the array, the dilution rates sequentially decreased. When all vessels were full, and it became obvious from the analysis performed that different bacterial groups had colonised the vessels, i.e. utilisation of SO_4 in the top vessel, and the production of methane, coupled with the production and utilisation of VFA's in all the vessels, the flow rate was increased to 10mlh^{-1} and the systems were allowed to stabilise for 108 days. After stabilisation (indicated by specific levels of VFA, sulphate and methane see Section 4.3), systems A and B were contaminated with DMP and *o*-cresol respectively starting from the bottom vessels (A3 and B3) up, this allowed the analysis of each of the bacterial associations rather than contamination from the top vessel (A1 and B1),

which would have a knock on effect on the lower vessels and would not allow the analysis of individual bacterial groups.

In a previous study by Sulisti (1994), the top vessel was operated under batch conditions for 16 days to allow the bacteria in the inocula to acclimatise. In the present study, it was decided to shorten this period and allow the spatial separation of the bacterial groups to the lower vessels of the system to occur sooner, by doing this it was hoped that a better segregation of the bacterial groups into separate vessels could be achieved. This was done to try and eliminate methanogenic bacteria establishing in the top vessel, as they have the ability to form biofilms, which could interfere with the study. This did not appear to work however, and some methane was recorded in the top vessels. Methane was also recorded by Sulisti (1994) and Holmes *et al*., (2002) in the top vessels of the systems.

All vessels in both system arrays were monitored at regular intervals for changes in methane and VFA concentrations using methods outlined in Sections 2.2.4 and 2.2.6. The pH was also analysed and the sulphate concentration in the top vessels of each system was monitored (Sections 2.2.1 and 2.2.2). After contamination of the systems with o-cresol and DMP via the three-way stopcock at the top of each vessel, samples were taken to monitor the concentrations of DMP and *o*-cresol to ensure that they remained constant (Section 2.2.5). Due to the continuous flow of the systems the concentrations of DMP and o-cresol were constantly diluted. Therefore this was checked daily and further additions of the compounds were made.

In order to stop bacterial wash out of the systems, which had been observed in the laboratory previously by Sulisti (1994), the flow rate of the pumps providing medium to the systems was set at a lower rate than previous experiment by Sulisti (1994). The previous studies had utilised landfill refuse as a source of bacterial inocula (Sulisti 1994; Holmes *et al*., 2002), the lower flow rates ensured that washout of the sulphate reducing bacteria, acetogenic bacteria and methanogens did not occur in these systems.

Before the analysis of the effects of o-cresol and DMP on the specific bacterial groups, the systems had to stabilise over time, this allowed a clearer picture of the spatial separation of the bacterial groups and which vessels in the systems they inhabited.

Once it was clear that bacterial groups had established themselves in different vessels of the systems, and a stabilisation pattern could be seen throughout both of the systems, DMP and *o*-cresol were introduced to systems A and B respectively. Contamination with these compounds began at the bottom vessel of each system A3 and B3 to allow the assessment of each the compounds toxicity on the spatially separated groups in the systems.

Introducing the phenolic compounds to the lowest vessels of the systems allowed analysis of their effects on specific bacterial groups that were present in those particular vessels to be carried out without inhibition of substrate generated by bacteria present in the upper vessels of the systems. If the perturbation of the systems with these compounds had started at the top vessels, then the exact effect on different bacterial groups could not have

been assessed due to the overflow from vessel to vessel down the array and the compounds would have inhibited bacterial processes which produced substrates for groups in vessels further down the systems.

Over time increasing concentrations of DMP and *o*-cresol were added to vessels A3 and B3 respectively (Table 4.1). The concentrations of each compounds added were 0.6mM, 1.2mM, 2mM and 2.5mM DMP and 1.9mM, 2.8mM, 3.7mM and 4.6mM *o*-cresol. Contamination concentrations were based on the results from the previous batch culture experiments (chapters 3), the lowest concentrations were chosen due to them having inhibitory effects on the batch cultures. After three complete volume changes the concentrations were increased. Increases continued until the complete inhibition of bacterial species being studied was observed. This was when the methane production reached zero. When complete inhibition was observed the next vessel up the array was then perturbed with *o*-cresol or DMP. The experiment was stopped for vessels A1 and B1 when it was clear inhibition of sulphate reduction had occurred.

Table 4.1 Concentrations of DMP and o-cresol added to vessels A3 and B3

Time (Days)	A3 [DMP] mM	B3 [*o*-cresol] mM
108	0.6	1.9
168	1.2	2.8
222	2.0	3.7
270	2.5	4.6

4.2.2 Genomic DNA extraction

All sample centrifugation was carried out at 4°C.

A 50 ml sample was extracted from the system vessel and centrifuged for 10 minutes at 3000rpm immediately. The supernatant was then removed using a pipette. 20 ml of PBS was used to wash the pellet, the solution spun at 3000rpm for 5 minutes at 4°C and the supernatant was again discarded. The pellet was suspended in 500µl of lysozyme (4mg/ml), transferred to a 1ml Eppendorf tube and incubated for 30 minutes at 37°C. After the incubation period 250µL of 2% Sodium dodecyl sulphate (SDS) containing 1mg/ml proteinase K was added and the solution was incubated for a further 60 minutes. 250µl phenol/chloroform (1:1), was then added and the tube was inverted a few times until the solution went white, it was then spun in a micro-centrifuge for 2 minutes. This stage separated the solution into 2 layers. The top layer containing the DNA was collected and the bottom layer containing the proteins and cellular material not needed was discarded. The phenol chloroform stage was then repeated until there was no white

precipitate at the interface of the 2 layers. The top layer was then collected and 3M sodium acetate pH 5.4 (0.1 volume) and isopropanol (1 volume) added; the solution was then mixed and left on ice for 10 minutes. The tube was then spun in a micro-centrifuge for 10 minutes, the supernatant discarded and the pellet was allowed to air dry for approximately 40 minutes. To the dry pellet 45μL of TE containing 4mg/ml RNAase was added to resuspend the pellet. The DNA was stored at 4°C

4.2.3 Polymerase Chain Reaction (PCR)

The volume of each reaction in the PCR experiments was 100μL. The 100μL reaction contained 20μL of 5 X Go Taq buffer containing 7.5mM $MgCl_2$ (Promega, Southampton, UK), 1μL of 10mM dNTPs mix (Promega, Southampton, UK). 1μL of forward and 1μL of reverse primer, at a final concentration of 1pmol, were added to each reaction along with 61μL of sterile H_2O and 5μl of DMSO. 10μL of template "bacterial DNA" was added to each reaction and a negative control was included containing 10μL of sterile water instead of template DNA. Finally, 0.25μL (1.25U) of GoTaq® DNA Polymerase (Promega, Southampton, UK) was added to each reaction. In the case of the archaea PCR reaction 5 μl acetimide was added to eliminate unspecific binding.

4.2.4 PCR Conditions

Both bacterial and archaeal conditions and the primers used were taken from (Nakatsu *et al*., 2000). The bacterial primers used were PRBA338F and PRUN 518R. The archaeal primers were PRA 46F, PREA 1100R, PARCH 340F and PARCH 519R. The primer sequences can be seen in Table 4.2.

Table 4.2 PCR primers for the specific amplification of the bacterial or archaeal 16S gene.

Primer name	16S rDNA target (base number)*	Primer sequence
PRBA338F	Bacteria V3 region (338-358)	5' AC TCC TAC GGG AGG CAG CAG 3' **
PRUN518R	Universal V3 region (534-518)	5' ATT ACC GCG GCT GCT GG 3'
PRA46F	Archaea (46-60)	5' C/TTA AGC CAT GCG/A AGT 3'
PREA1100R	Archaea (1117-1100)	5' T/CGG GTC TCG CTC GTT G/ACC 3'
PARCH340F	Archaea V3 region (340-358)	5' CC TAC GGG GC/TG CAG/C CAG 3' **
PARCH519R	Archaea V3 region (534-519)	5' TTA CCG CGG CG/TG CTG 3'

*** Bases numbered relative to E. coli 16S rRNA sequence**
**** 5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G 3'**
(Nakatsu *et al*., 2000)

4.2.4.1 Bacterial PCR conditions

The PCR cycling conditions were as follows: DNA denaturation at 94°C for 9 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. This was completed with an extension step of 72°C for 7 min. The primers used in this amplification were PRBA338F and PRUN 518R.

4.2.4.2 Archaea PCR conditions

The amplification of 16S archaeal DNA was carried out using a nested PCR technique, adapted from Nakatsu *et al*., (2000). The conditions for the first amplification were: DNA denaturation at 92°C for 5 min, followed by 30 cycles of 92°C for 30 s, 53.5°C for 30 s, and 72°C for 1min and a final extension step of 72°C for 5min, using the primers PRA 46F and PREA 1100R. The second round amplification was the same as the first only the annealing temperature was dropped to 53°C, the extension time decreased to 30 s and the primers were changed to PARCH 340F and PARCH 519R.

4.2.5 Agarose gel electrophoresis

PCR products were visualised using gel electrophoresis. A 1.5 (w/v) agarose (Bioline Ltd., London, UK) gel in 1 × TAE was used to run samples due to the small size of the PCR amplicons. 1.5g of agarose (Bioline Ltd., London, UK) was added to 100mL of 1 × TAE buffer in a 250mL conical flask. The flask was heated in a microwave until the agarose had dissolved. The molten agarose was allowed to cool to 50°C, 2µL of ethidium bromide (10mg/mL) or sybrsafe (Invitrogen, Ltd, Paisley, UK) was added and the agarose was poured into a gel cast. The gel was allowed to set for 1 h, it was placed in a BRL H5 gel tank (BRL/Life technologies, UK) and covered in 1 × TAE running buffer. Samples were loaded onto the gel using 2 × DCode (Biorad, Hertfordshire, UK). A 100bp DNA ladder (Promega, Southampton, UK) was used to determine the size of small PCR amplicons. The DNA samples were separated by electrophoresis for 40 min with an applied current of $5Vcm^{-1}$. At the end of this time gels were photographed on a UV transilluminator at 302nm using a Kodak DC200 digital camera and Kodak Digital Science ID Image Analysis Software (Kodak, Hemel Hempstead, UK).

4.2.6 Gel extraction

Due to unspecific primer binding in the archaeal PCR, the correct size products were excised and PCR was performed again to show only one 200b.p. product prior to DGGE. DNA bands were extracted from the agarose gels stained with sybr green (Invitrogen, Paisley, U.K.) and visualised using a dark reader (Clare Chemical Research, Colorado, U.S.A.). The DNA was then extracted using a QUAEX II DNA extraction kit (Qiagen, West Sussex , U.K.).

4.2.7 Denaturation gel electrophoresis

DGGE was carried out using a Bio-Rad d-code universal mutation system (Bio-Rad, Hemel Hempstead, U.K.); the gels contained 8% bis/acrylamide and were poured following the manufactures guidelines. The denaturation gradient of the gel was 30% - 60%. To produce this gradient two gel solutions had to be prepared containing differing percentages of urea and formamide; the gel constituents can be seen in Table 4.3.

Table 4.3 gel denaturation solutions.

Denaturation	30%	60%
40% Acrylamide/Bis	20ml	20ml
50 x TAE buffer	2ml	2ml
Formamide (deionised)	12ml	24ml
UREA	12.6g	25.2g
dH_2O	To 100ml	To 100ml

Immediately before the gels were poured, final concentrations of 0.09% (v/v) each of ammonium persulfate and TEMED were added to the solutions to allow the gel to set.

Once the denaturation gel had been prepared the PCR products were injected into the gel wells. Electrophoresis was run at 10V for 20 min then at 200V for 6 to 8 hours in 1x TAE buffer. The gel tank was maintained at a 60°C throughout the run. The gels were then placed in 250ml of running buffer containing 10mg/ml ethidium bromide (50µg/ml) and stained for 20 min. The stained gels were photographed on a UV transilluminator at 302nm using a Kodak DC200 digital camera and Kodak Digital Science ID Image Analysis Software (Kodak, Hemel Hempstead, UK).

4.3 Results from stabilisation of systems

Figures 4.1-4.8 show the stabilisation of the systems over time, for pH, methane and VFA's. It is apparent from analysis of the stabilisation charts from both systems A and B that there are slight differences in the results. These show the unpredictability of working with biological systems, as both the systems and inocula were similar. Raw data can be found in Appendix II.

4.3.1 Sulphate

Sulphate concentration in the input media was 4.1mM. During the system stabilisation period no sulphate was detected in the top vessel of either of the systems, with the exception of system failure or malfunction due to media contamination. System malfunction was initially thought to be bacterial washout due to flow rates. After investigation, it was bacterial growth was discovered in the medium inlet tubing. This growth utilised the carbon source intended for the top vessels of the systems, therefore sulphate was not utilised as an electron acceptor due to the decrease of carbon entering the top vessels, it therefore appeared that bacterial washout had occurred due to sulphate concentrations remaining high in the top vessels of the systems. Input medium contamination occurred frequently at the beginning of the experiment, due to this the medium was changed every 3-4 days, contamination was also noted on the tubing carrying the medium to the top vessel, this contamination was not obvious and had to be detected by close observation of the tubing and was initially overlooked and the systems were thought to have had bacterial washout. To combat this tubing was changed every week as cleaning and autoclaving did not seem to prevent the growth.

System failure was also noted due to the formation of Struvite crystals under anaerobic conditions these crystals are magnesium ammonium phosphate and are common in anaerobic digestion due to the release of ammonium and phosphate. These crystals would congregate in the y-tube connectors between vessels causing blockage and affecting the flow off the systems due to media backing up to the upper vessels.

The fact that no sulphate was detected in these vessels under normal running conditions indicates that all of the sulphate is being utilised by the sulphate reducing bacteria. This result showed that sulphate reducing bacteria had colonised the vessels of the systems. In a similar studies by Sulisti (1994) and Holmes *et al.,* (2002), it was noted that the majority of sulphate reduction occurred in the top vessel, but some sulphate was still detected in the second vessels. In the present study no sulphate was detected in the top vessels indicating complete utilisation of this electron acceptor. The difference in sulphate utilisation between this study and the other two could be due to the higher flow rates used in both ($30mlh^{-1}$ c.f. $14mlh^{-1}$in this study) and the differences in the inocula used. In this study the inocula was an active batch culture containing return activated sewage sludge, where as previous studies by Sulisti (1994), James *et al.,* (1998) and Holmes *et al.*, (2002) used the more active landfill inocula. The complete utilisation of sulphate in the top vessels may also indicate unbalanced growth conditions which could allow methanogenesis in this vessel as mentioned in Section 4.2.1 Future experiments should include an increase in sulphate concentrations within the medium as this would possibly control the Redox potential of the vessels and inhibit the growth of methanogenic bacteria. In

addition to more sulphate, less carbon source could be added. It is possible that if there is an abundance of carbon source then sulphate reducing bacteria and methanogens can co-exist, if there is less carbon source then the sulphate reducing bacteria will outcompete the methanogens. This is due to sulphidogenisis having a lower Gibbs free energy (-62.9 $kJmol^{-1}$) than methanogenisis (-47.4 $kJmol^{-1}$), therefore sulphate reducing bacteria use less energy per mole of hydrogen utilised (Lupton and Zeikus, 1984).

4.3.2 pH

The pH of systems A and B differ between vessels (Figures 4.1 and 4.2). An increase in pH was observed down both the system's arrays, with the highest pH being noted in the bottom vessels A3 and B3. The difference in pH between vessels of a system was due to the production and utilisation of VFA. It can be seen from Figures 4.4 – 4.9 that the majority of VFA's were produced in the top vessel of the systems, thus lowering the pH of these vessels. These acids were then utilised in the lower vessels with maximum utilisation occurring in the bottom vessel, where utilisation of the acids was indicated by an increase in pH .

Comparison of the pH charts obtained from both systems inoculated with the same culture and run under equal conditions showed that a similar pattern developed in both for pH. While slight differences were noted between the systems the pH of both appears similar.

The pH of all vessels did not dramatically change due to the buffering capacity of the input media.

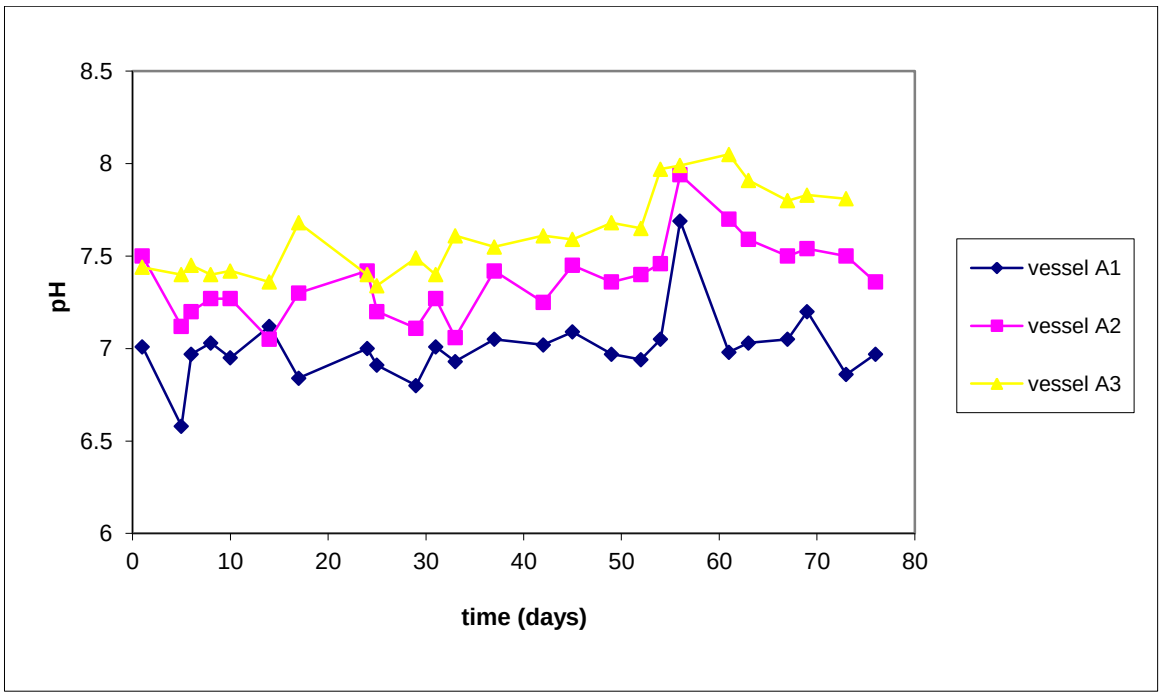


Figure 4.1 pH versus time stabilisation chart for system A

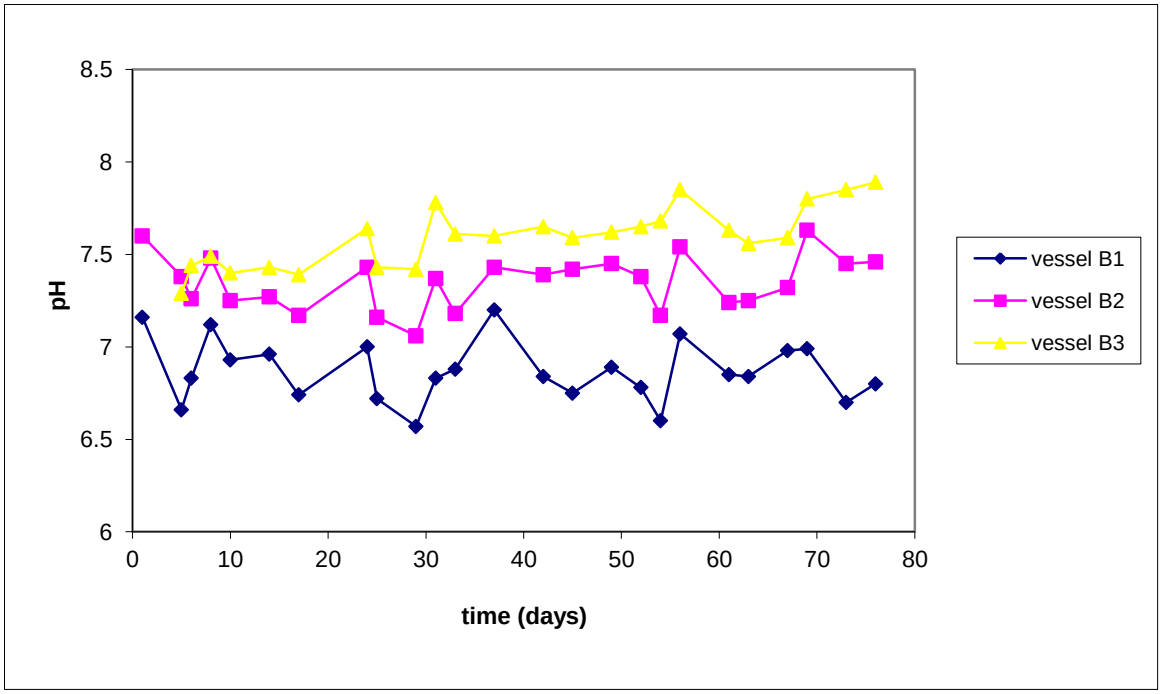


Figure 4.2 pH versus time stabilisation chart for system B

4.3.3 VFA

As shown in Chapter 3, the breakdown of cellobiose by the anaerobic bacterial consortium produced the VFA's acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate and hexanoate, with acetate, propionate and butyrate being the most abundant. Figures 4.4 to 4.9 show the concentrations of acetate, propionate and butyrate down the systems array, the values obtained for the remaining 4 acids were very low. Concentrations and figures for these have been placed in Appendix II.

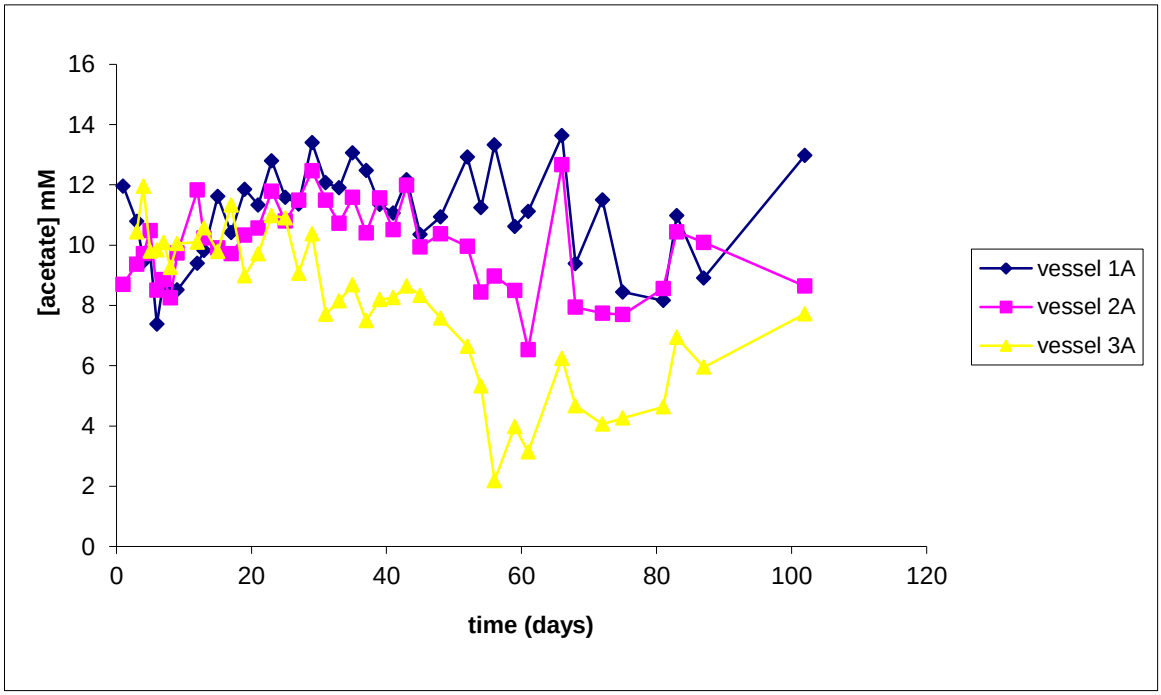


Figure 4.3 Acetate versus time stabilisation chart for system A

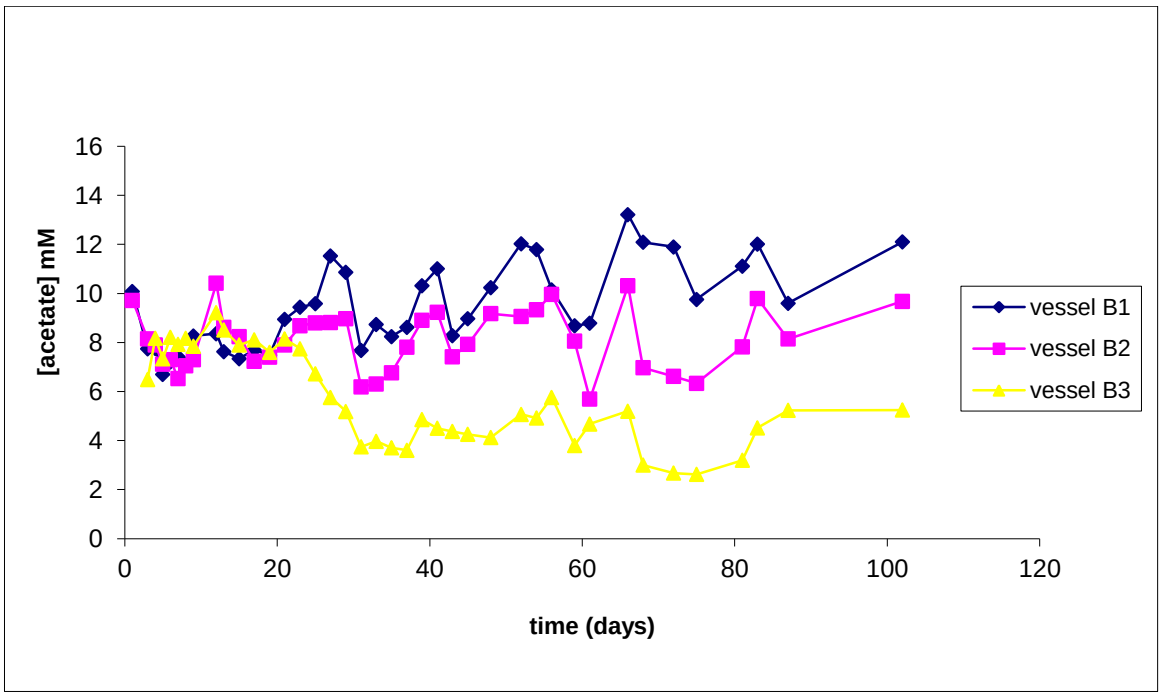


Figure 4.4 Acetate versus time stabilisation chart for system B

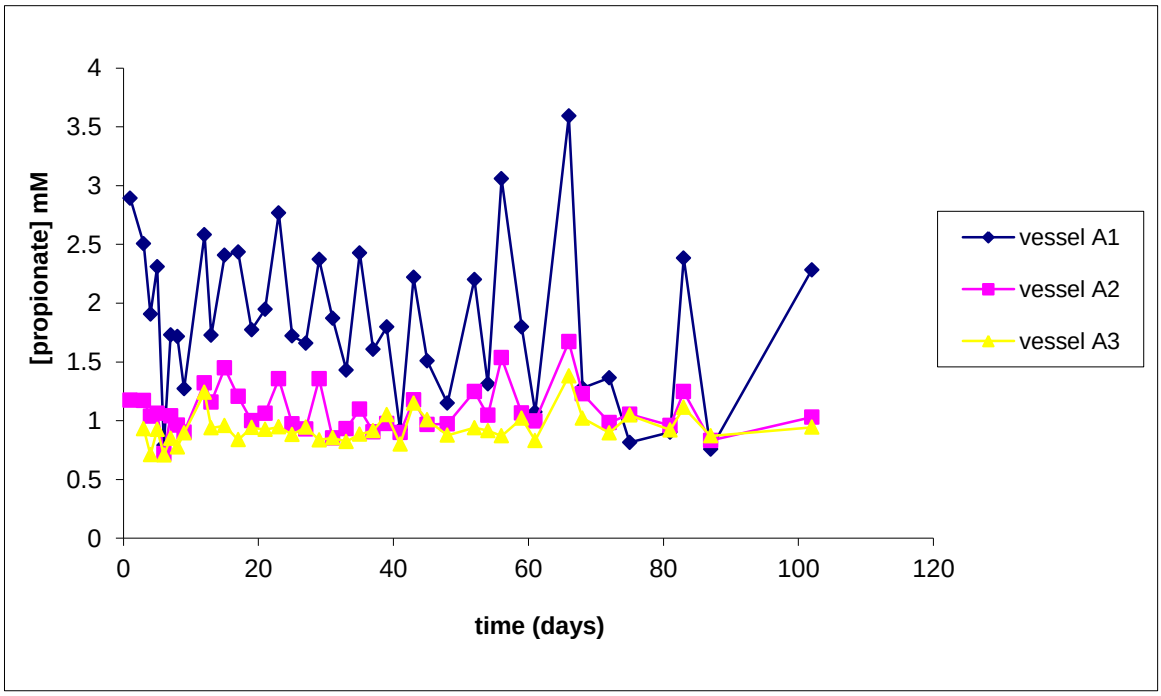


Figure 4.5 Propionate versus time stabilisation chart for system A

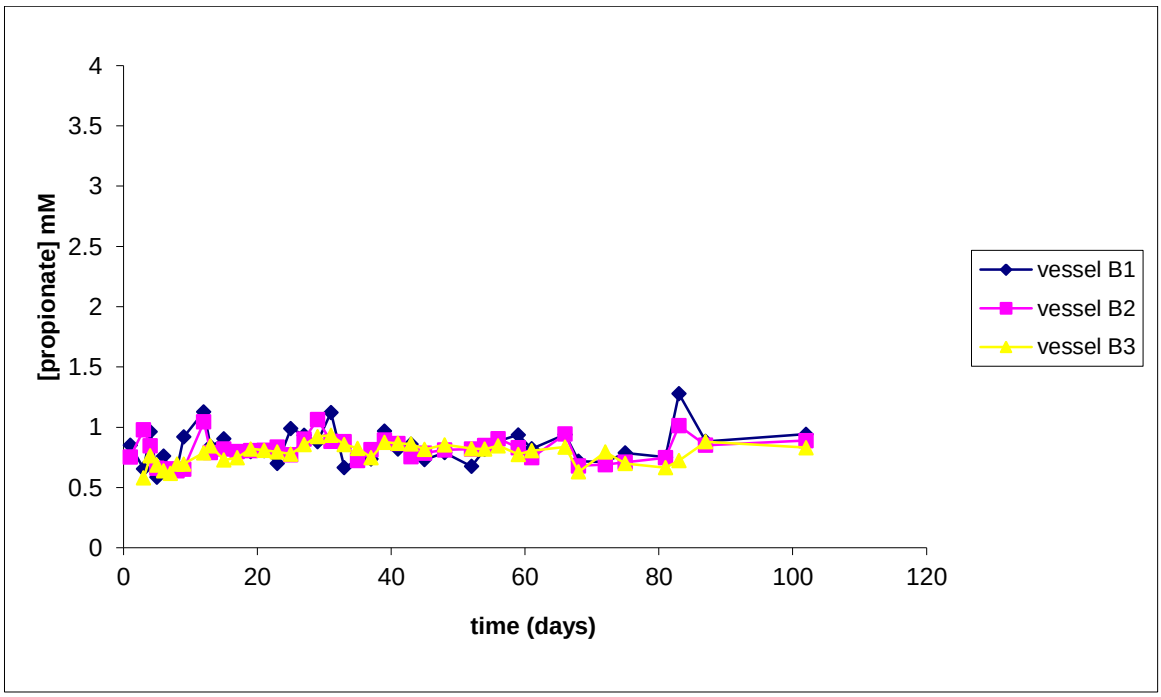


Figure 4.6: Propionate versus time stabilisation chart for system B

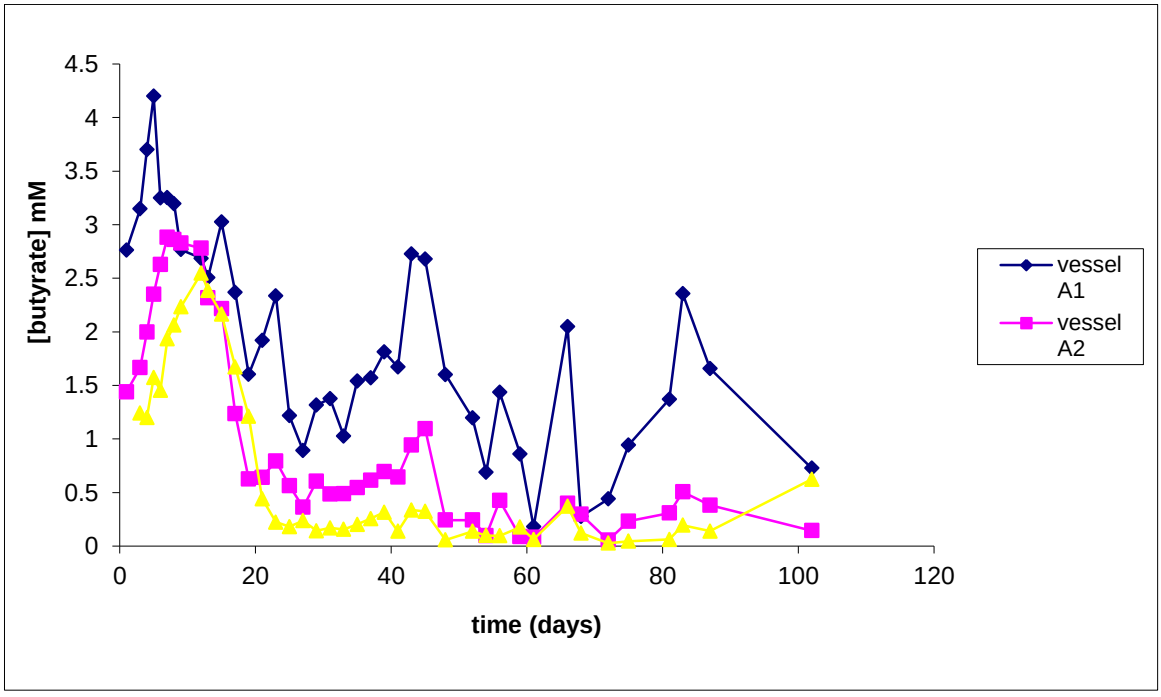


Figure 4.7: Butyrate versus time stabilisation chart for system A

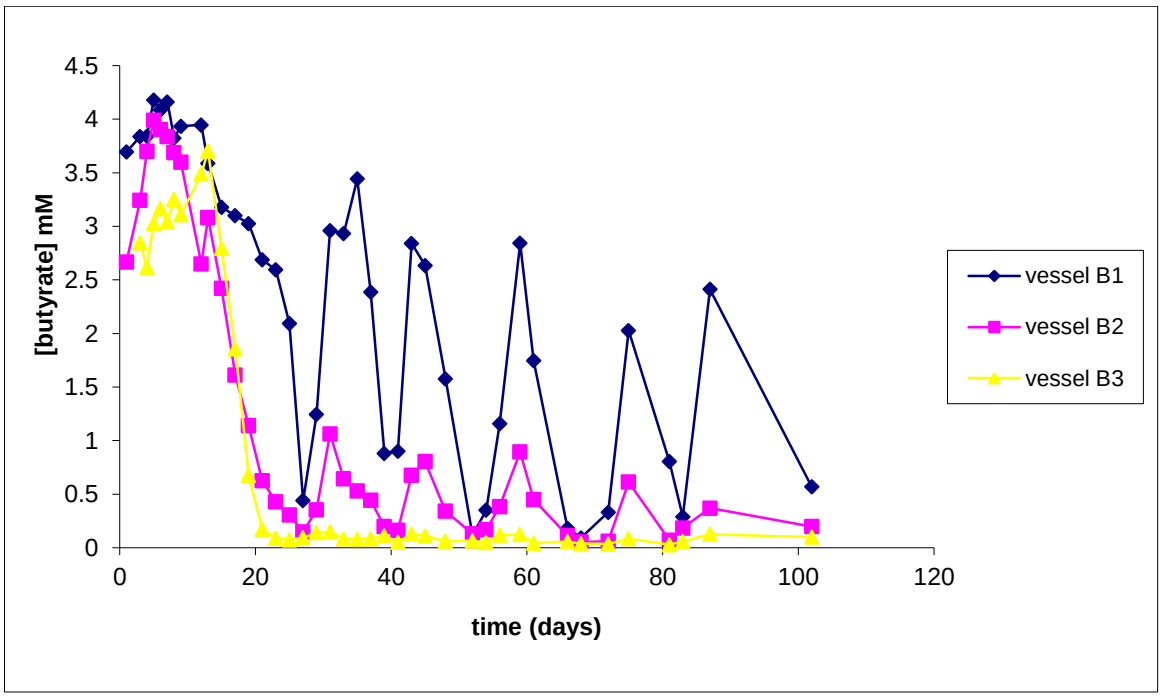


Figure 4.8: Butyrate versus time stabilisation chart for system B

Acetate concentrations in both systems decreased down the vessels (Figures 4.3 and 4.4.). The reduction of acetate concentration is an indication that acetate was utilised. The highest concentrations were observed in vessels A1 and B1. Since no acetate was present in the input medium, it can be concluded that acidogenic VFA producing bacteria and acetate producing acetogenic bacteria were responsible for the acetate generated, indicating that these bacterial groups have established themselves in the top vessels of the systems. As the medium, now containing acetate progressed down the systems the concentration of acetate was seen to decrease from vessels 1 to 2 only a slight reduction in acetate concentration was noted, indicating very moderate utilisation of this acid and possibly more production. The acetate concentrations in the third vessels of the systems are lower than the top

vessels, which showed that the majority of acetate utilisation occurred in these vessels.

All the VFA's concentrations with the exception propionate decreased down the systems array indicating that in the lower vessels the bacterial groups present utilised these acids to produce acetate, although it might also be possible that the acids were being produced and utilised at a similar rate. As previously stated in Chapter 3, under normal conditions it is not energetically favourable for anaerobic bacteria to utilise propionate and butyrate. The utilisation of these VFA's only occurs when the hydrogen partial pressures are reduced to less than 2×10^{-3} atm and 9×10^{-5} atm for butyrate and propionate respectively (McInerney and Byrant, (1981)), this occurred when other bacterial groups utilised the hydrogen, for example hydrogenotrophic methanogens and SRB. Therefore as shown in Chapter 3, the bacteria that utilised propionate and butyrate must have existed in a synotrophic relationship with the H_2 utilising bacteria.

Figures 4.5 and 4.6 show the propionate concentrations of all the vessels of systems A and B. The propionate concentrations in system A were at their highest in the top vessel, a reduction in this concentration was observed in the second vessel and only a slight insignificant reduction was observed from vessel A2 to A3. The reduction in propionate concentration from the top to the second vessel was due to utilisation of the acid to produce acetate, indicating that the hydrogen partial pressure has been reduced enough to make propionate reduction energetically favourable, only slight reduction occurred from the second to the third vessel indicating that the majority of the propionate utilising bacteria are present in vessel A2. As previously

mentioned the lack of acetate utilisation coupled with methane production shows that hydrogen utilising methanogenic bacteria inhabit this vessel allowing for the hydrogen partial pressure to be reduced allowing for propionate reduction. Although reduction of propionate was observed from vessel s A1 to A2 it was never fully utilised, possibly due to the number of bacteria which inhabit these vessels or possibly nutrients becoming the limiting factor, or that the end product of propionate utilisation was acetate and hydrogen which inhibited propionate degradation by lowering the available free energy for the degradation reaction (Voolapalli and Stuckey (1999)).

The propionate concentrations in system B were lower than those found in system A and did not appear to reduce down the system array, since both systems A and B were the same specification and the inocula was from the same culture, this can only be put down to the problems associated with working with mixed culture populations and the fact that although the systems were set up to replicate one another, differences in bacterial activity may have occurred.

The conditions in the systems allow the breakdown of butyrate. This can be seen with the analysis of Figures 4.7 and 4.8. In the previous batch culture studies (Chapter 3), the breakdown of butyrate was only noted in the controls after 30 days, therefore the systems set up which allows for the spatial separation of nutrients and distinct microniches of bacterial groups forming in the different vessels, enabled the utilisation of this VFA due to the creation of more energetically favourable conditions, as mentioned in the introduction Section 1.3. The breakdown of nutrients and use of electron acceptors alters the redox conditions. In the systems set up, each vessel down

the array will have different redox conditions and different hydrogen partial pressure allowing some substrates to be used in one vessel, but not another. The reduction of H_2 partial pressure allowed the breakdown of firstly butyrate and then propionate as mentioned in Section 3.3.8. In the lower vessels of both systems, the butyrate concentration was reduced with the lowest levels noted in the bottom vessels. The maximum butyrate reduction in both of these systems occurred in the second vessels and appeared to be coupled to the production of methane by hydrogenotrophic methanogens.

4.3.4 CH_4

Figures 4.9 and 4.10 show the methane produced in each vessel. Due to the complex set up of the systems and the constant flow of oxygen free nitrogen into each vessel, it was impossible to perform headspace analysis for methane gas, if it had been possible continuous methane monitoring would have been preferred, but due to the restraints of laboratory equipment, gas chromatography was the only technique available. The amount of soluble methane in each vessel was therefore used as an indicator of methane concentration, but not the total amount of methane produced.

Analysis of soluble methane concentrations of the vessels of both systems clearly indicates that the amount of methane produced increased down the vessels of the systems. By analysing both the methane and VFA charts a clearer picture can be seen of which bacterial groups have established in the systems vessels.

The top vessels of both systems produce methane which indicates that the methanogenic bacteria are co-existing in this vessel alongside SRB's this coexistence was previously noted by Sulisti (1994) Parkes *et al*., (2005) and Conway de Macario and Macario (2008), although it was previously thought that the coexistence of these two bacterial groups could not occur due to the SRB's out competing the methanogens for substrates and H_2. Raskin *et al* (1996) stated SRB's will out-compete methanogens in the presence of a non-limiting sulphate supply, in the case of this experiment, the sulphate is constantly being pumped into the vessels, but it is utilised immediately which would allow the methanogens to utilize the excess H_2. As indicated in Section 4.3.1, the concentration of sulphate entering the top vessels may have been too low, this would possibly allow more reduced redox conditions to occur. These conditions may have been more favourable for the growth of the methanogenic bacteria. Since in these systems there is a constant flow of fresh nutrients into the top vessels, the competition between bacterial groups may be alleviated. Methanogenic bacteria are also known to attach to surfaces and form biofilms (Yu *et al*., 1992, Hidalgo and Garcia-Encina, 2002). As the inocula of both systems was a methanogenic batch culture then the already batch established methanogens may have quickly established themselves to the surface of the top vessels. The fact that methanogenic bacteria were active in the top vessels shows that complete separation of the different bacterial groups was not completely achieved. Possibly increasing the initial flow rate after batch stabilisation or decreasing the amount of time allowed for batch stabilisation, coupled with an increase in sulphate present in the input medium could allow for better separation. From analysis of the stability results it can be seen that the different vessels down the array

contain different dominant processes, therefore they have different redox conditions, which allow different species to dominate.

Analysis of the methane production and VFA, specifically acetate utilization, of all the vessels in the systems determined that the majority of the methane produced in the top two vessels of the systems was from the activity of hydrogenotrophic methanogens. This was indicated by the apparent lack of acetate utilization, although some of the methane produced in these vessels may be attributed to acetoclastic methanogens from the analysis of the methane and VFA figures this was not clear.

The bottom vessels of the systems produced the highest methane concentrations and concentrations of the acetate were at their lowest indicating that acetoclastic methanogens had established themselves in these vessels and were utilizing acetate to produce methane.

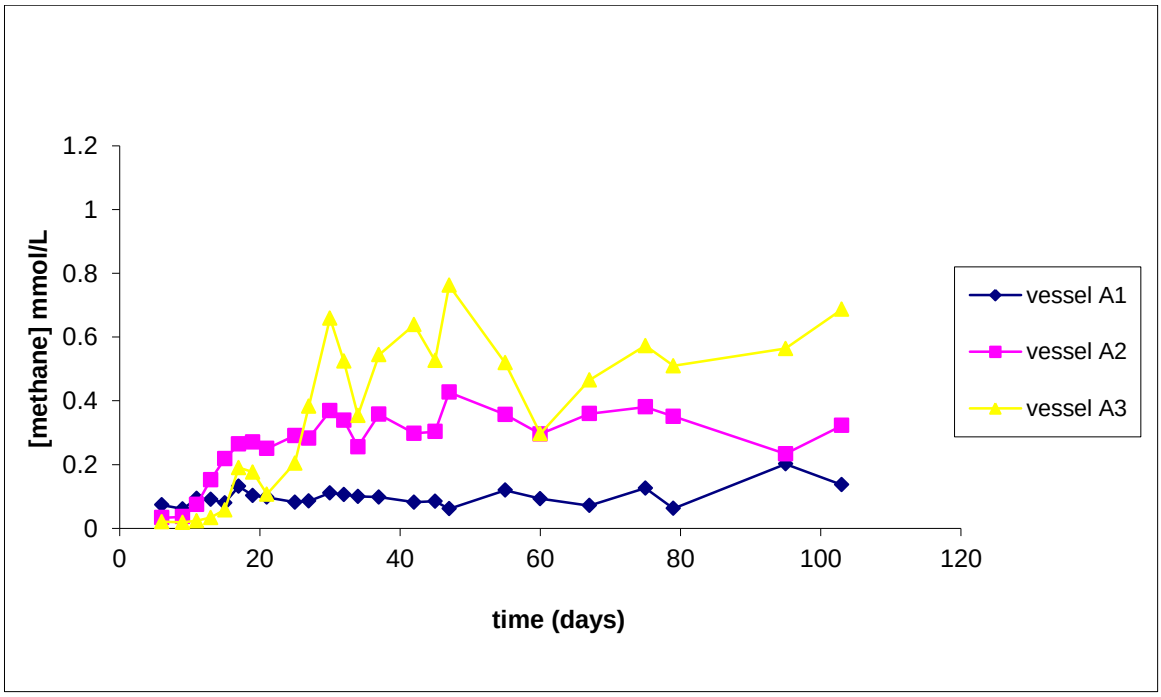


Figure 4.9 Methane versus time stabilisation chart for system A

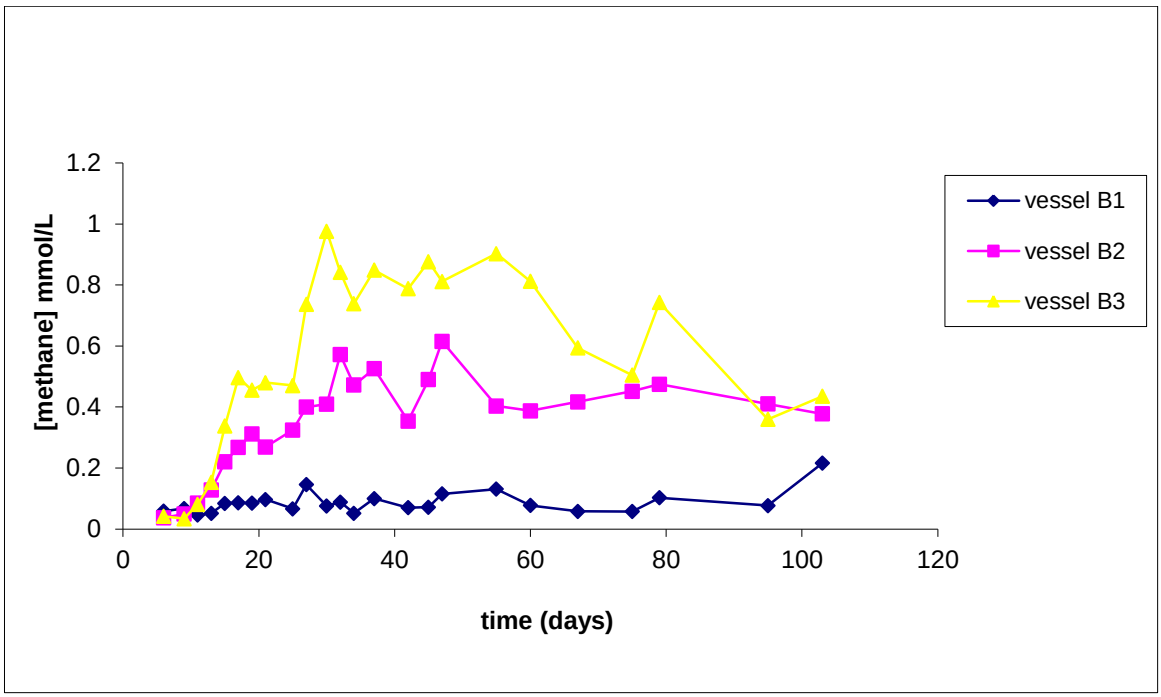


Figure 4.10: Methane versus time stabilisation chart for system B

After both systems had stabilized (108 days), it was clearly seen that the different bacterial groups in the consortium were separated by nutrient flow rates into distinct habitat domains in the separate vessels, with overlapping activity domains. Figure 1.5 shows the separation of the bacterial groups within a system although not completely accurate as there appears to be an overlap of bacterial between the vessels i.e. methanogenisis in the top vessels the main groups that are abundant in the vessels are highlighted in the figure.

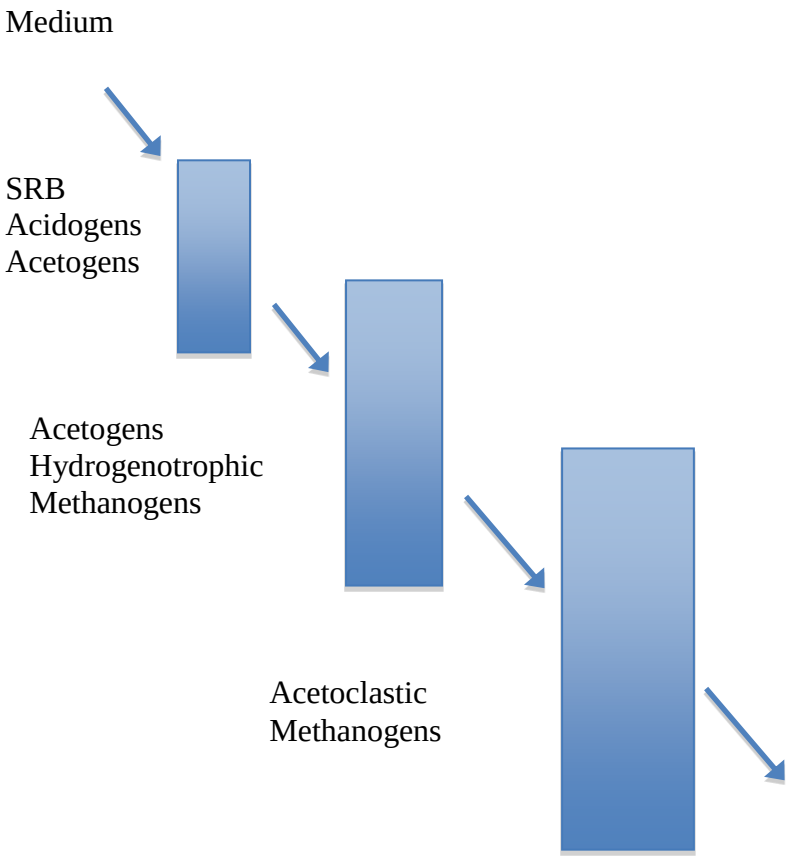


Figure 1.5 Schematic diagram of the main bacterial separation in systems (repeated from chapter 1).

4.4 Contamination of systems results and discussion

4.4.1 Vessels A3 and B3

Vessel A3 and B3 were contaminated with DMP and *o*-cresol respectively (see Table 4.1).

Figure 4.11 shows the methane concentration over time for both vessels with the addition of the varying concentrations of the alkylphenols.

All the concentrations of DMP and o-cresol added to vessels A3 and B3 respectively over time caused a decrease in methane production. Due to the sensitive nature of the systems, shutdown occurred due to input medium contamination when this occurred the systems were allowed to settle and re-establish with no further addition of DMP or o-cresol, which in turn affected the concentrations of methane produced and the phenolic contaminants (see section 4.3.1). When the systems re-established the phenolic compounds were reintroduced and their concentrations were kept constant.

With the addition of 0.6mM DMP to vessel A3 and 1.9mM o-cresol to vessel B3 an initial drop in methane produced was noted in both vessels indicating the inhibition of methanogenic bacteria. Since methane was still being produce the concentrations of both DMP and o-cresol were increased to 1.2mM and 2.8mM respectively. At 1.2mM of DMP vessel A3 showed an initial increase in methane production, which then levelled off and remained constant indicating the possible development of the methanogenic bacteria's adaptation to DMP, which could be due to the long exposure time to the lowest concentration (0.6mM). The methane production in vessel B3, after exposure to an *o*-cresol concentration of 2.8mM, showed an initial increase in methane followed by a sharp drop to approximately 0.2mmol/L by day 185

from this day until day 207 the methane level decreased only slightly. At the third increased addition of both DMP and o-cresol (concentrations 2mM and 3.7mM respectively) the methane production in both vessels was greatly reduced, to lower than the concentrations caused by previous additions of the contaminants, indicating that although the bacteria in these vessels may have developed a tolerance to the phenolic compounds at these levels the increased concentrations of the alkylphenols greatly reduced methane production due to the possible inhibition of the methanogenic bacteria. On day 270 vessels A3 and B3 were perturbed with 2.5mM DMP and 4.6mM of o-cresols respectively. These concentrations of the contaminants caused a rapid decrease in methane production, which then ceased altogether indicating complete inhibition of the methane producing archaea. After complete inhibition was noted no further *o*-cresol or DMP was added and a recovery of methanogenic activity was noted indicating that the bacteria were inhibited, but not killed.

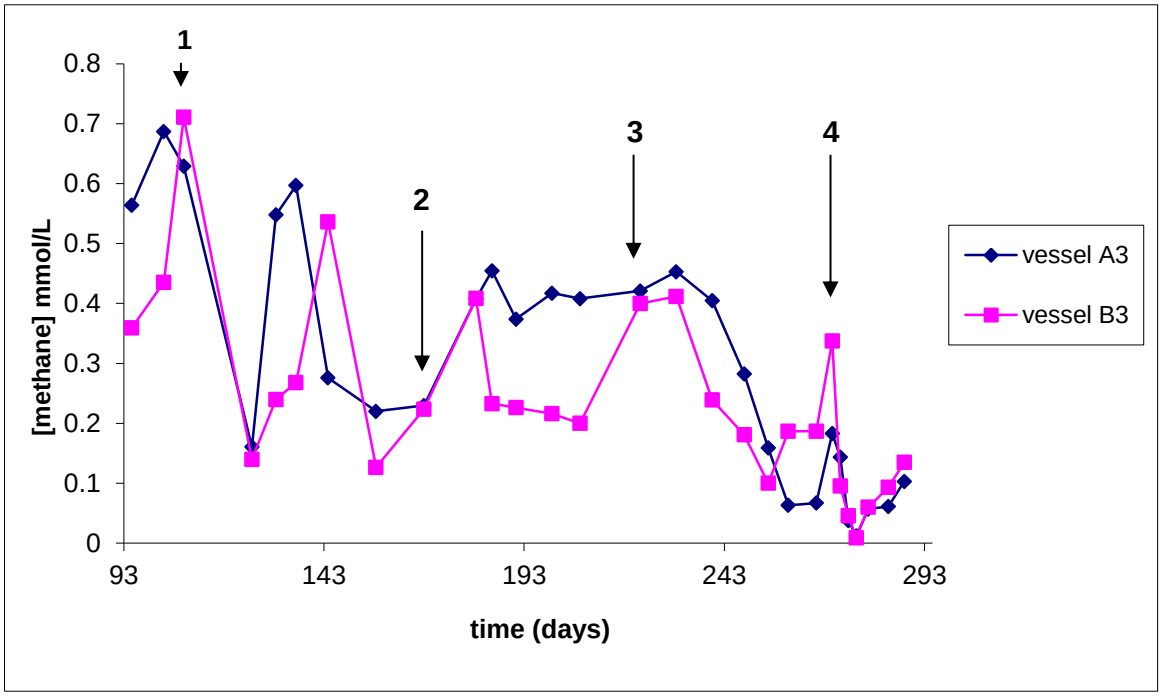


Figure 4.11: Methane versus time for vessels A3 and B3.

The numbered arrows correspond to the addition of (1) 1.9mM *o*-cresol or 0.6mM 2,4DMP; (2) 2.8mM *o*-cresol or 1.2mM 2,4DMP; (3) 3.7mM *o*-cresol or 2mM 2,4DMP; (4) 4.6mM *o*-cresol or 2.5mM 2,4DMP.

4.4.2 VFA

Of all the VFA monitored, the acetate concentration was the most relevant to this study. As shown previously in this chapter the second highest VFA concentrations were propionate and butyrate, but due to the low levels of these two acids, the differences noted in the concentrations as a result of contamination of the vessels with the two phenolic compounds were too small to be useful. Therefore although all seven VFA's were monitored only the acetate is shown for system contamination, the rest of the VFA concentrations can be found in Appendix II.

With the addition of DMP and *o*-cresol to vessels A3 and B3 respectively the acetate levels increased, although as previously mentioned in this chapter due to problems with the day to day maintenance of the systems the acetate levels dropped due to the contamination of input media and system shutdown. This in turn caused the DMP and *o*-cresol concentrations to drop when the systems were re-establishing due to there being no further additions of the phenolic compounds until the systems stabilised. Analysis of Figure 4.12 shows that the addition of the lowest concentrations of DMP and o-cresol caused an increase in acetate concentrations. This indicates a possible reduction in utilisation of acetate with production being possibly

unaffected. With the addition of DMP and *o*-cresol the acetate levels initially drop and then increase indicating an initial inhibition of the acidogenic bacteria, which may be present in these vessels hence causing a reduction in acetate production. At the highest concentrations of DMP and *o*-cresol (2.5mM DMP and 4.6mM *o*-cresol) acetate concentrations in the vessels increase rapidly indicating an inhibition of acetate utilisation.

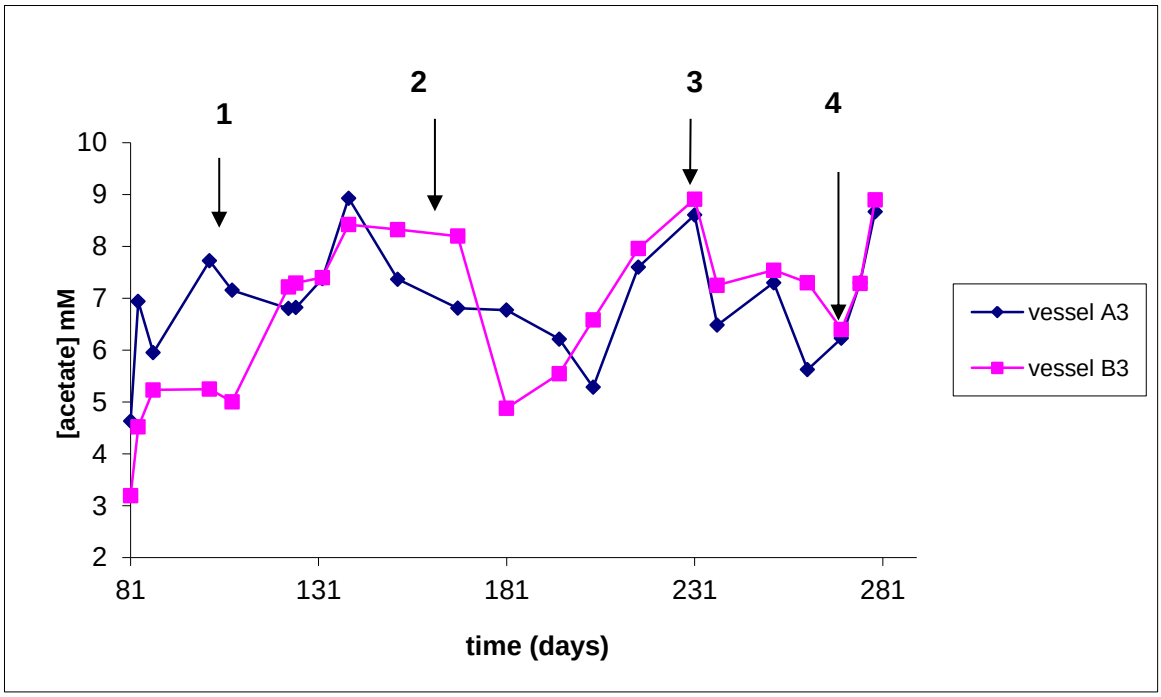


Figure 4.12: acetate versus time for vessels A3 and B3.

The numbered arrows correspond to the addition of (1) 1.9mM *o*-cresol or 0.6mM 2,4DMP; (2) 2.8mM *o*-cresol or 1.2mM 2,4DMP; (3) 3.7mM *o*-cresol or 2mM 2,4DMP; (4) 4.6mM *o*-cresol or 2.5mM 2,4DMP.

Coupling the acetate results with the results obtained from the methane analysis, it can be clearly seen that all the concentrations of DMP and *o*-cresol have an inhibitory effect on methane production and acetate utilization

indicating inhibition of acetate utilizing acetoclastic methanogens. At the highest concentrations used 2.5mM DMP and 4.6mM *o*-cresol, complete inhibition of methane production and the greatest increase in acetate production was noted, indicating complete inhibition the acetoclastic methanogens that dominated these vessels.

4.4.3 pH

Figure 4.13 shows the pH against time for vessels A3 and B3 during the contamination period. The pH over the contamination period in vessels A3 and B3 showed no change at the two lowest concentrations of DMP and *o*-cresol. This result was also observed in previous studies (Sulisti, 1994). At the highest concentrations of the phenolic compounds a slight reduction in pH was observed due to the possibly increase in acetate concentration.

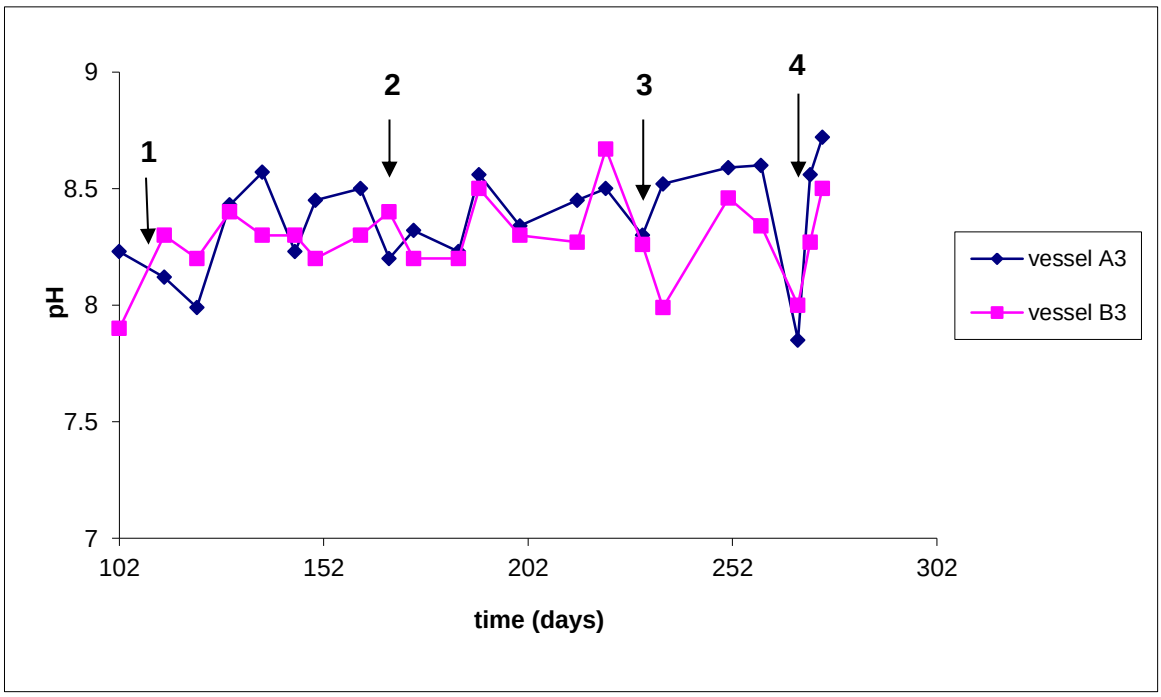


Figure 4.13: pH versus time for vessels A3 and B3.

The numbered arrows correspond to the addition of (1) 1.9mM *o*-cresol or 0.6mM 2,4DMP; (2) 2.8mM *o*-cresol or 1.2mM 2,4DMP; (3) 3.7mM *o*-cresol or 2mM 2,4DMP; (4) 4.6mM *o*-cresol or 2.5mM 2,4DMP.

4.4.4 Vessels A2 and B2 Methane

All concentrations of DMP added to vessel A2 and all concentrations of o-cresol added to vessel B2 appeared to have exhibited an effect on methane production. As the concentrations of the contaminants increased, the methane production in both vessels decreased. On analysis of Figure 4.14 it was observed that the introduction of 1.9mM o-cresol into vessel B2 had a greater effect on methane production than the introduction of 0.6mM DMP into vessel A2.

Increasing the concentrations of DMP to 1.2mM decreased the methane concentration to below 0.1mmol/L, with further increases in DMP the methane concentration remained constant indicating a possible tolerance due to increased exposure to the contaminant. When vessel A2 was perturbed with the maximum concentration of DMP (2.5mM), methane production ceased completely indicating inhibition of the methanogenic bacteria in this vessel.

The initial addition of the lowest concentration of *o*-cresol (1.9mM) produced a sharp decrease in methane production from 0.2mmol/L to 0.07mmol/L. A further though significantly smaller decrease was noted with the addition of 2.8mM from 0.07mmol/L to 0.03mmol. The methane concentration stayed at this value after the addition of 3.7mM o-cresol showing that the bacteria may have developed a tolerance to *o*-cresol. Complete inhibition of methanogenic bacteria was observed with a further increase of o-cresol to 4.6mM.

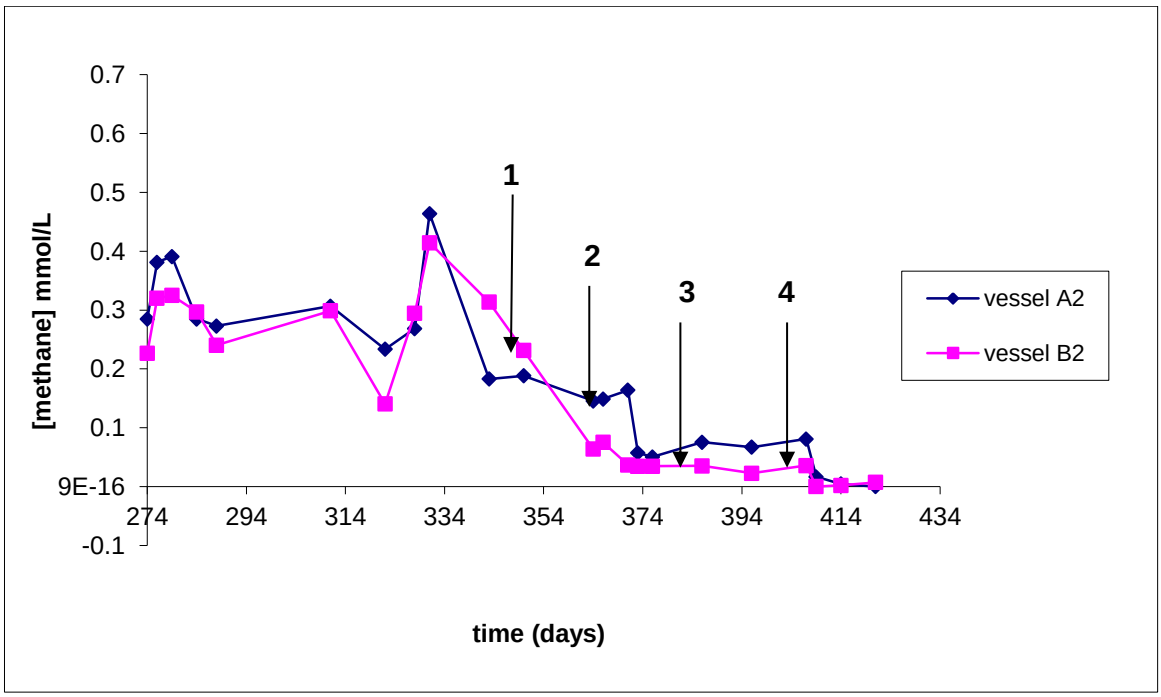


Figure 4.14 methane versus time for vessels A2 and B2.

The numbered arrows correspond to the addition of (1) 1.9mM *o*-cresol or 0.6mM 2,4DMP; (2) 2.8mM *o*-cresol or 1.2mM 2,4DMP; (3) 3.7mM *o*-cresol or 2mM 2,4DMP; (4) 4.6mM *o*-cresol or 2.5mM 2,4DMP.

4.4.5 VFA

As described in the previous Section 4.4.2, the most relevant VFA acid studied in this experiment was acetate, all other VFA results can be found in Appendix II.

The addition of all concentrations DMP and *o*-cresol to vessels A2 and B2 appeared to have no effect on the acetate concentrations of these vessels. This may be an indication that this acid was not utilised or produced in this vessel (Figure 4.15) or that there was a rapid turnover of acetate utilisation and production. From these results it appears that methane production in these vessels may be independent of acetate concentration. This indicates that the predominant methanogenic bacterial group, which inhabit the second vessels of both systems, are the hydrogenotrophic methanogens, which combine CO_2 and H_2 to produce methane.

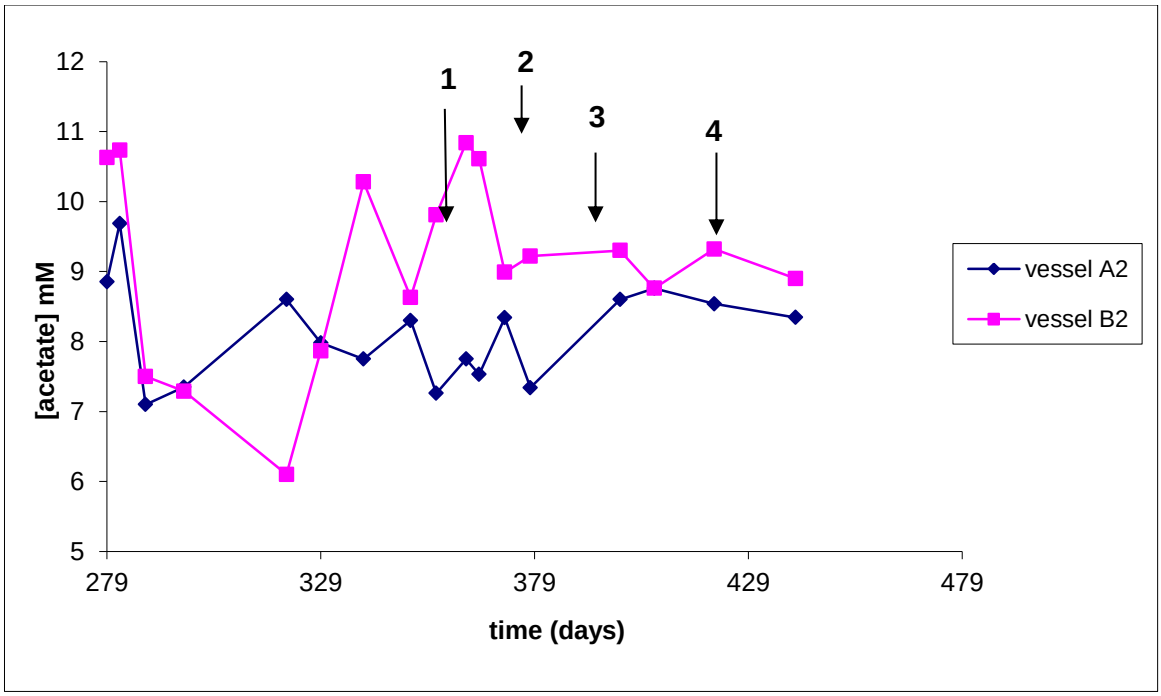


Figure 4.15: Acetate versus time for vessels A2 and B2.

The numbered arrows correspond to the addition of (1) 1.9mM *o*-cresol or 0.6mM 2,4DMP; (2) 2.8mM *o*-cresol or 1.2mM 2,4DMP; (3) 3.7mM *o*-cresol or 2mM 2,4DMP; (4) 4.6mM *o*-cresol or 2.5mM 2,4DMP.

4.4.6 pH

The pH in vessels A2 and B2 showed slight fluctuations (Figure 4.16) during the course of the contamination, but this appeared to be independent of contamination. As the contamination of these two vessels did not appear to affect the VFA concentrations in these vessels there was no increase in acetate due to the predominant bacterial species in these vessels being hydrogenotrophic methanogens. As previously mentioned the input media has a good buffering capacity therefore, even if an increase in acid concentration was noted, the pH would remain stable as it did in vessels A3 and B3.

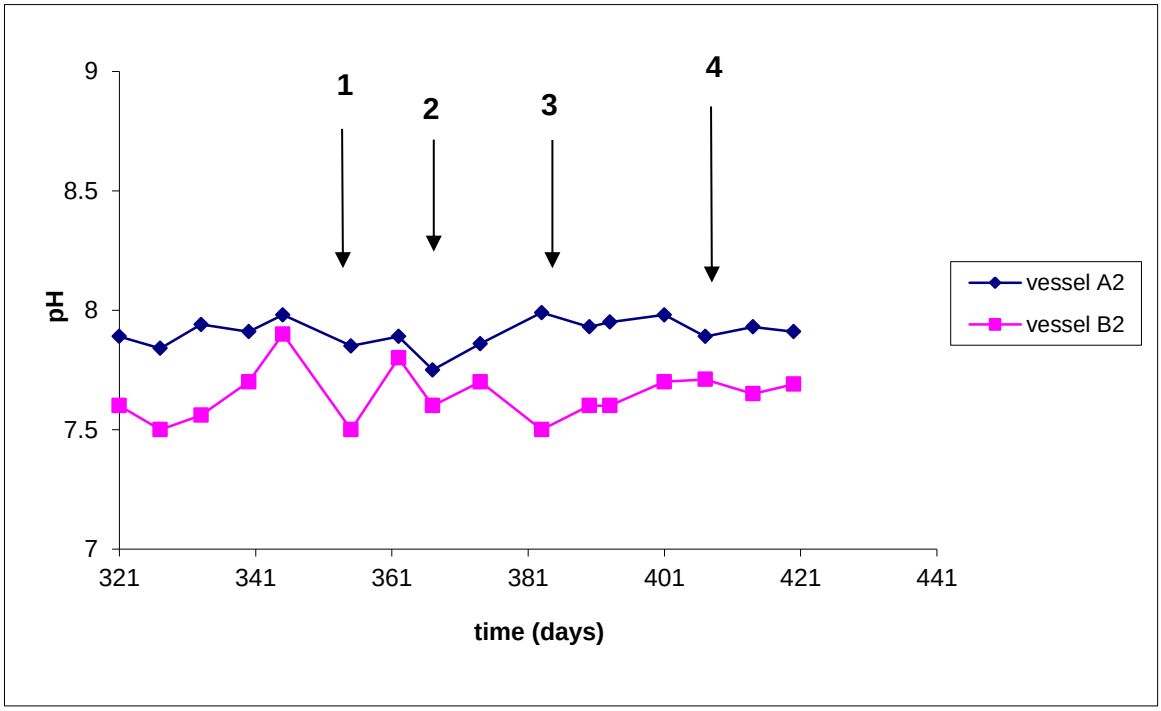


Figure 4.16 pH versus time for vessels A2 and B2.

The numbered arrows correspond to the addition of (1) 1.9mM *o*-cresol or 0.6mM 2,4DMP; (2) 2.8mM *o*-cresol or 1.2mM 2,4DMP; (3) 3.7mM *o*-cresol or 2mM 2,4DMP; (4) 4.6mM *o*-cresol or 2.5mM 2,4DMP.

4.4.7 Vessels A1 and B1

The top vessels of the systems are where the input media enters the systems; these vessels contain the SRB. Therefore with no inhibitory compounds added all sulphate entering these vessels is completely reduced. From the stabilization graphs (Figs 4.1-4.10) it was shown that these two vessels contained the highest concentrations of VFA's, the lowest pH, produce some methane and reduce all the sulphate present in the input media. The bacterial group of interest in these vessels was the sulphate reducing bacteria. During the stabilization period, no sulphate was detected in these vessels, except when system failure occurred.

Figure 4.17 shows the sulphate concentrations of vessels A1 and B1 during the course of the phenolic contamination. On day 456 vessels A1 and B1 were perturbed with 0.4mM DMP and 1.9mM *o*-cresol respectively causing a slight increase in SO_4 concentration by the next day. From day 448 to day 449 the SO_4 concentration increased rapidly to 0.85mM in vessel A1 and 0.9mM in vessel B1. Between days 449 and 455 the sulphate concentration in both vessels decreased but never reached zero. On day 456 the concentrations of DMP and *o*-cresol in vessels A1 and B1 were increased to 0.8mM and 2.8mM respectively, with this increase a gradual increase in sulphate concentration was noted and by day 461 the sulphate concentrations in vessels A1 and B1 were 0.9mM and 1.01mM respectively. The concentrations of DMP and *o*-cresol were further increased on day 462 to 1.2mM and 3.7mM respectively with this increase in contaminant concentration a rapid increase in sulphate concentration was noted in both vessels by day 465 the sulphate concentrations in vessels A1 and B1 were 5.41mM and 5.85mM respectively.

At these levels of sulphate it was clear that the sulphate reduction had been completely inhibited.

On analysis of Figure 4.18 it can be seen that throughout the contamination period for vessels A1 and B1 the acetate concentration did not appear to be affected.

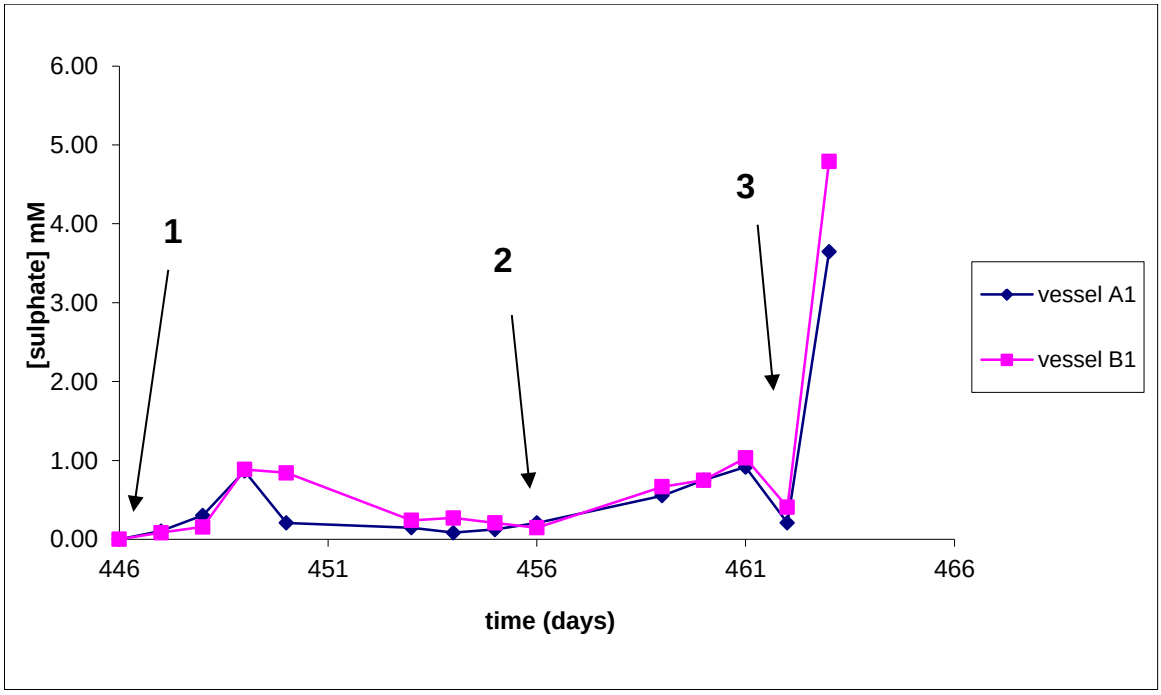


Figure 4.17: Sulphate concentration versus time for vessels A1 and B1.

The numbered arrows correspond to the addition of (1) 1.9mM *o*-cresol or 0.4mM 2,4DMP; (2) 2.8mM *o*-cresol or 0.8mM 2,4DMP; (3) 3.7mM *o*-cresol or 1.2mM 2,4DMP.

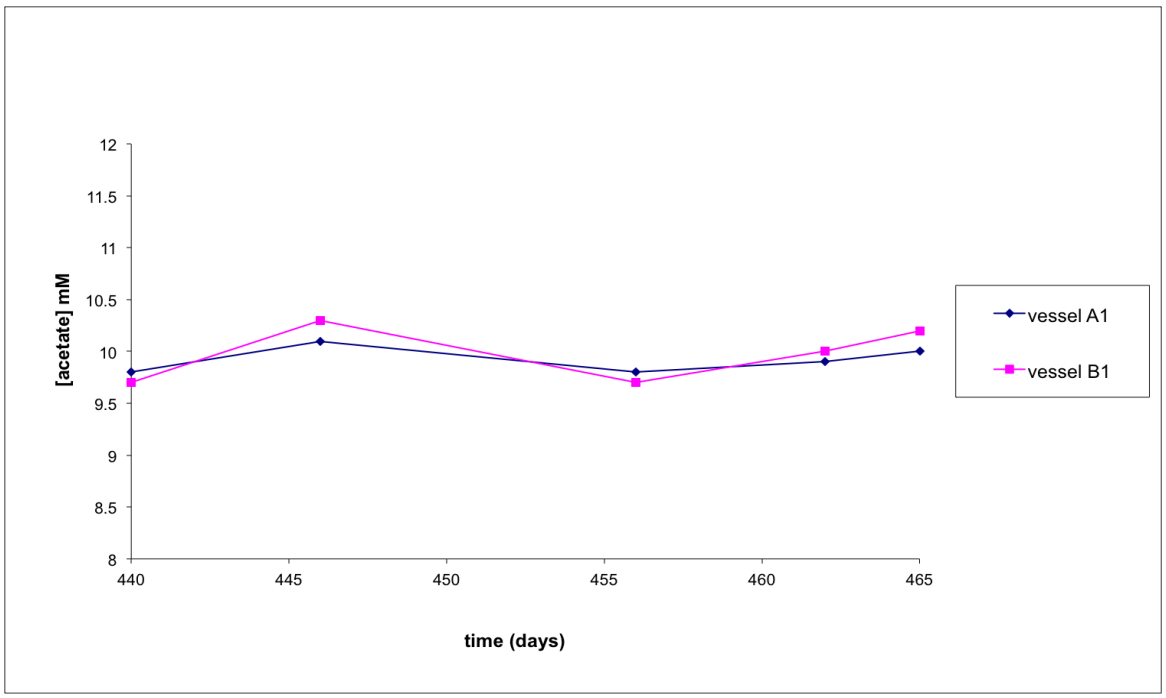


Figure 4.18: Acetate concentration versus time for vessels A1 and B1.

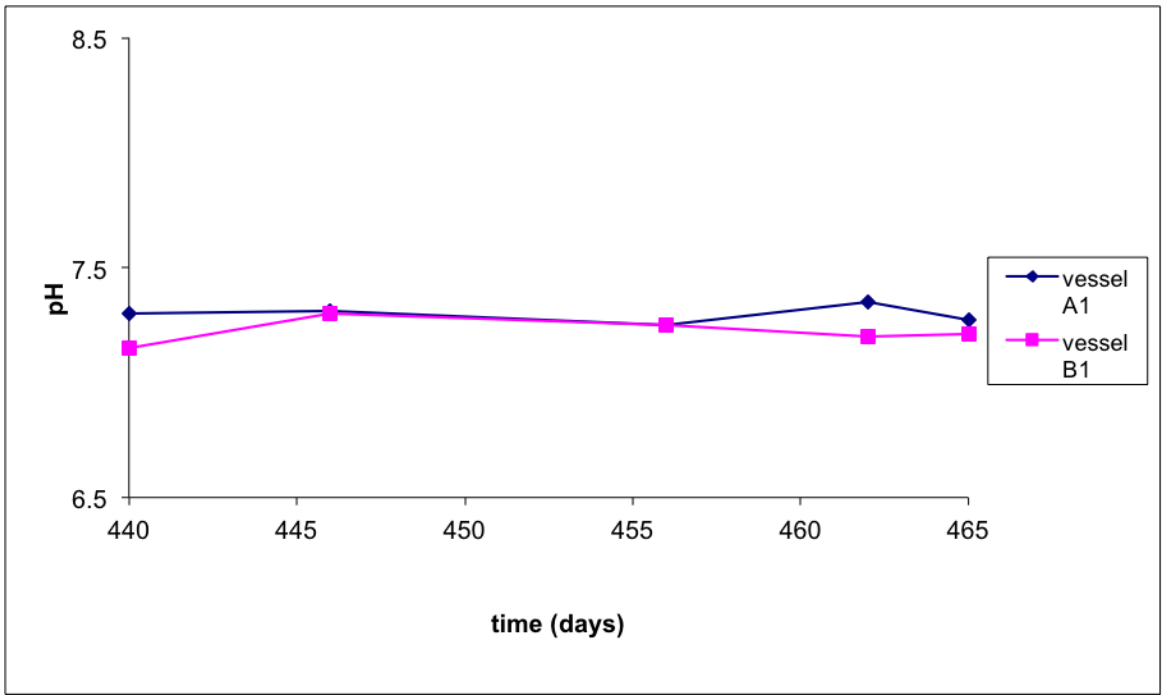


Figure 4.19: pH versus time for vessels A1 and B1.

The highest concentrations of DMP and *o*-cresol added to vessels A1 and B1 respectively appeared to have completely inhibited SRB and no effect was observed on acetate concentration (Figure 4.18) or pH indicating (Figure 4.19)

that these levels of contaminants did not inhibit the VFA producing acidogens.

4.5 Molecular analysis

To further understand the separation of the bacterial groups in the multi-stage systems, molecular techniques were employed. These techniques were PCR with Denaturation Gradient Gel Electrophoresis (DGGE). The samples for molecular analysis were obtained from vessels within systems C and D.

4.5.1 Molecular introduction

16S rDNA is the DNA gene coding the 16S RNA subunit, this gene has highly conserved regions which are the same in almost all bacteria these allow for the amplification of the variable regions using universal primers designed to attach to the conserved regions (Tran and Rudney, 1996; Schmalenberger *et al*., 2001).
Archaea also have conserved regions but these differ from the bacterial ones although they are the same for most of the archaeal species.

The use of 16S universal primers allows the same region of bacterial or archaeal DNA to be amplified. This enables the amplification of DNA of many strains from mixed population cultures. It is known that the specific regions of 16S DNA i.e. V3 region vary in all bacterial species the fact that the DNA in either sides of this region has the same code allows for the amplification of these regions. The variable regions of DNA may only vary by one or two base pairs between bacteria. This variation allows identification methods to be employed, for example DNA sequencing. From

the results of this the DNA sequence can be compared to libraries of known sequences allowing the determination of the specific bacteria and archaea within environmental samples. DNA is extracted from the mixed cultures; to aid the investigation of these mixed populations it is necessary to separate the individual species. The extracted DNA is amplified using PCR, the products of the PCR are all approximately the same length (in this experiment 200 base pairs (b.p)) which only appears as one band on an agarose gel, because of this a technique for separating the different types of bacterial DNA is needed. The samples need to be run on a gradient gel, which will separate the different DNA. The gradient can be in the form of temperature or chemical.

The DGGE technique involves using a urea formamide denaturation gradient, which denatures the G-C bonds within the DNA. This causes the DNA to slow and eventually stop, the more G-C rich a sample the further it will run on the gel. The PCR products run on the gels contain mixed bacterial DNA therefore when the gel is finished running and visualised each band observed is a different bacterial species and the intensity of the band indicates that species predominance in the culture.

4.6 Experimental Procedure

Samples were extracted from two continuous flow three stage systems, these systems (C and D) had been set-up to analysis the effects of combined phenols on the bacteria consortia. The results from these systems did not enable the study of the combined effects of these compounds and were omitted. It was decided to use these systems for analysis of the populations utilising molecular techniques.

System C was contaminated with varying concentrations of *o*-cresol and DMP, while system D was set up as a control and received no contaminants.

50 ml samples were taken from the vessels of the continuous flow multi stage systems when differences in methane production were observed. DNA was extracted according to Section 4.2.2. The extracted DNA was amplified using PCR with universal bacterial and archaeal primers following the method Sections 4.2.4.1. and 4.2.4.2. Visualisation of the 200b.p PCR product was carried out on a 1.5% agarose gel (Figure 4.20) with lanes numbered 1,2 and 3 containing the PCR product and lane 4 containing a 100b.p. ladder. Non specific binding was observed in the PCR product containing archaeal DNA, even though acetamide had been added to the PCR mix as stated in the method Nakatsu *et al*., (2000), the lanes containing bacterial DNA had showen only one DNA product of 200b.p in size. PCR gradients were set up to try to enable specific binding of the archaeal DNA (Figure 4.21) with lanes numbered 2-8 containing the PCR product, no optimum PCR conditions were discovered therefore the products were run on a 1.5% agarose gel containing sybr-safe and illuminated on a dark reader and the 200b.p. band was excised. The DNA was then extracted from the excised band following Section 4.2.6, the extracted DNA was amplified and the product was again checked on 1.5% agarose gel which showed one band of 200b.p..

10ul of the DNA samples were run on a 30-60% denaturation gel as described in Section 4.2.7. The samples run on the denaturation gel were from the different vessels to observe population shifts between the vessels and to compare the populations between identically set up systems.

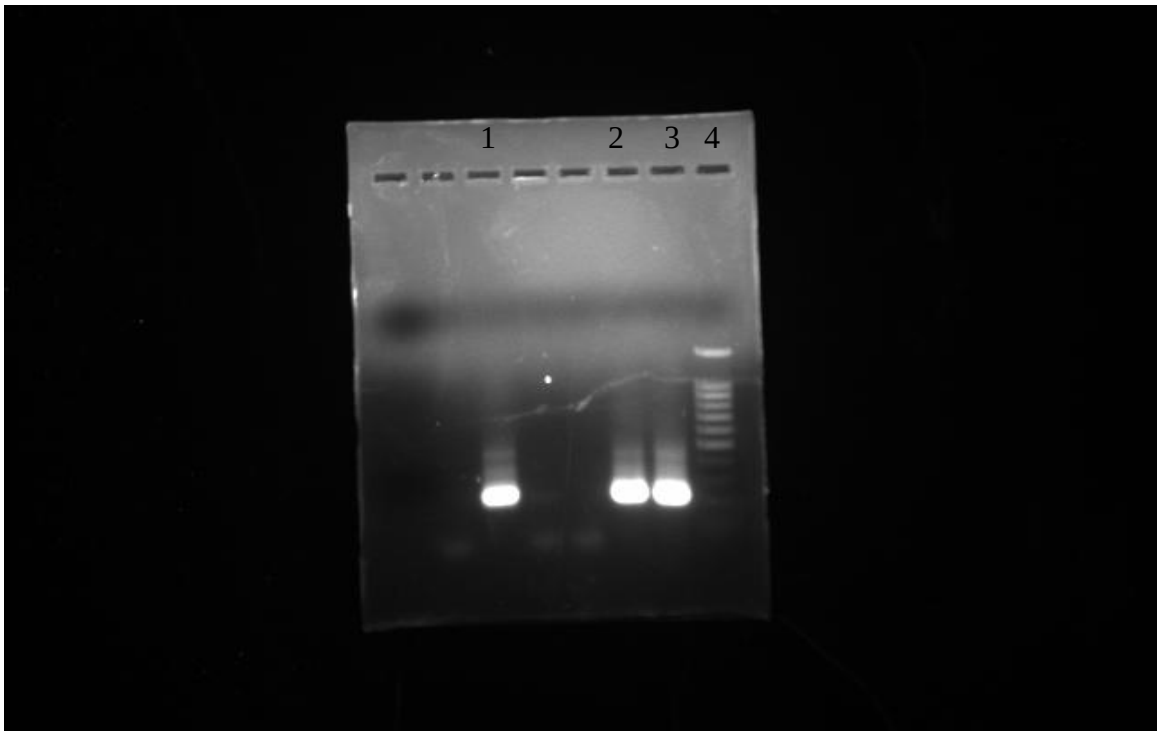


Figure 4.20 Agarose gel of Bacterial PCR product 200b.p. (lanes 1, 2 and 3), DNA ladder used for comparison was a Promega 100b.p (lane 4)

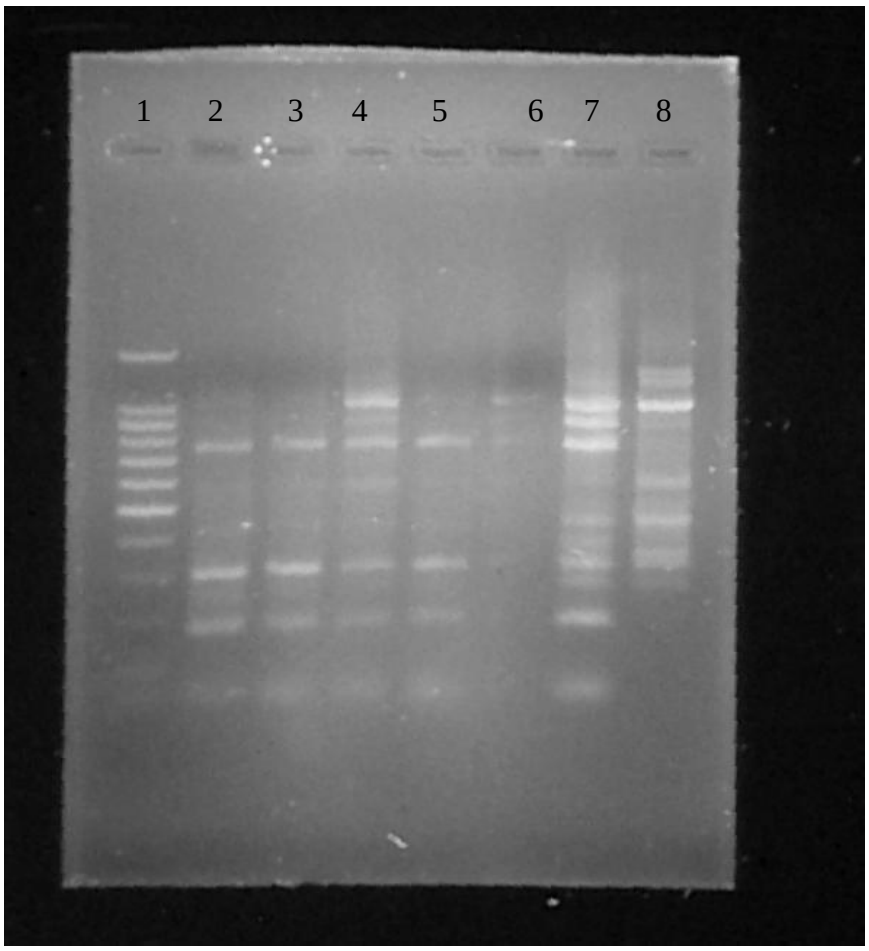


Figure 4.21-agarose gel of gradient PCR for archaeal DNA (lanes 2 – 8), the DNA ladder used for comparison was a Promega 100b.p (lane 1).

4.7 Results and discussion

Denaturation gradient gels were set up and bacterial and archaeal DNA were run. Figure 4.22 shows the DGGE gel stain for bacterial DNA. Each lane contains DNA from a different vessel of a system. At this point none of the systems had been contaminated with phenolic compounds and the systems had stabilised. From the analytical results obtained from the systems A and B e.g. sulphate reduction, VFA production and utilisation, and methane production, a picture of the different bacterial groups could be formulated; the predominant groups of bacteria in the vessels were known (Figure 1.5). Although as previously mentioned, the vessels of each system do have bacterial overlap. Each band on the gel represents a different type of bacterium. On analysis of the DGGE gel picture it can be seen that there are population differences between vessels, showing the spatial separation of nutrient distinct habitat domains achieved by 3 stage continuous flow systems. All five gel lanes have three close bands in the middle. These bands are faint in the lane 5 containing the DNA from vessel C1, but are still present. From the analytical methods used previously and the working knowledge of the systems, it was thought that these bands may be from the acetogenic bacteria, which were found in all vessels of the systems. Vessel C1 has other bands, which are seen in no other lane, these bands could be the sulphate reducing bacteria or fermentative bacteria (these are highlighted by arrows 1, 2 and 3). From the picture and the analytical results it was impossible to determine which bacterial group the bands come from, although the gel picture does show slight differences in populations between the vessels and also shows that the different systems appear to have the same populations as each other. Slight differences in bands can be seen between the systems, but the predominant bands are the same, arrows 4, 5, and 7

show the differences between the third vessels of both systems C and D, while arrow 7 highlights a band found in vessel D2 which is not present in vessel C2, although a similar band can be seen in vessel C3 (arrow 4). These differences in system populations are only slight and do not appear to affect the systems as a whole, but do show that even though they were run under the same conditions, it was not possible to use them as an exact comparison, due to the complex nature of the biological systems.

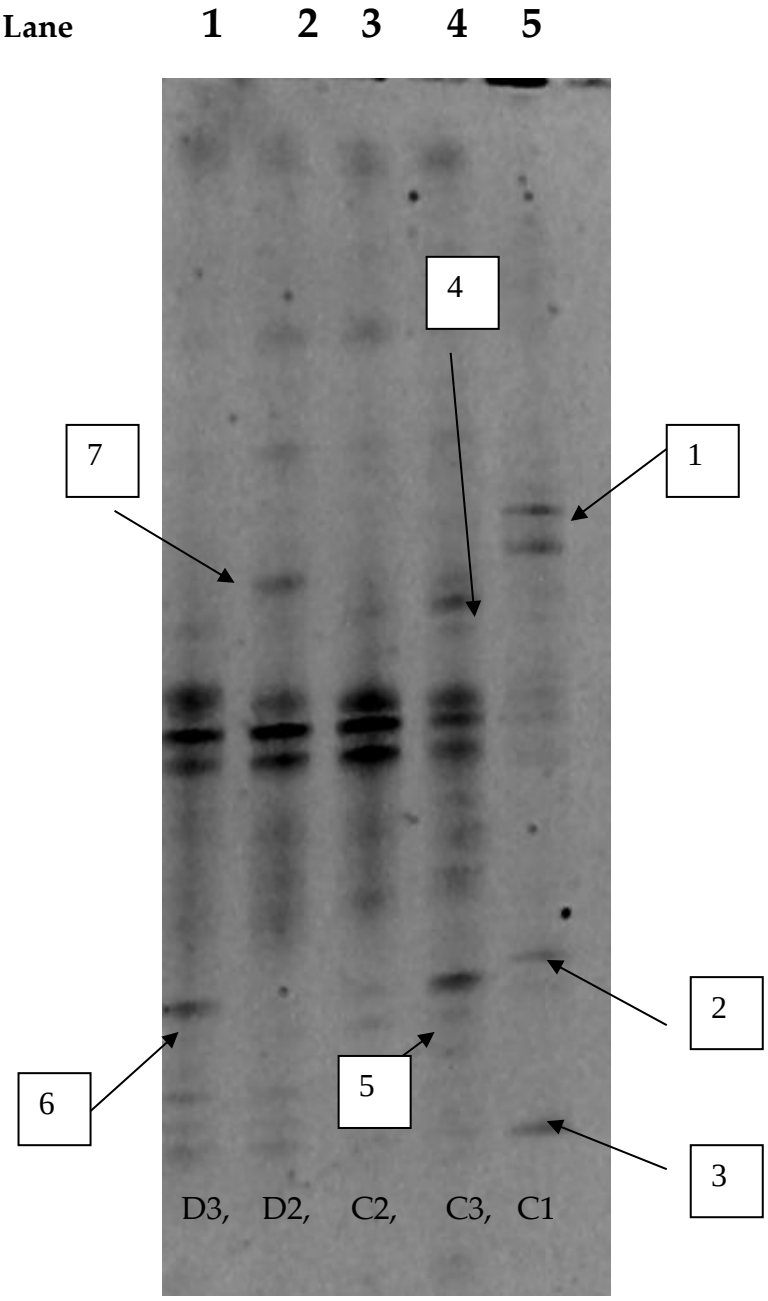


Figure 4.22. DGGE gel stain for bacterial DNA from systems C and D pre-contamination. (Possible sulphate reducing bacteria and fermentative bacteria are denoted by arrows 1, 2 and 3. Arrows 4, 5, and 6 indicate the differences between the third vessels of both systems C and D. Arrow 7 highlights a band found in vessel D2 which is not present in vessel C2 although a similar band can be seen in vessel C3 (arrow 4)).

After the initial study of bacterial populations using DGGE, system C was contaminated with combined concentrations of DMP and *o*-cresol (1.39mM DMP and 3.80mM *o*-cresol) on day 603. DNA was then extracted and amplified to enable the analysis of population shifts as a result of contamination. Figure 4.23 shows the methane production for vessels C3 and D3, showing that the methane production in vessel C3 was reduced.

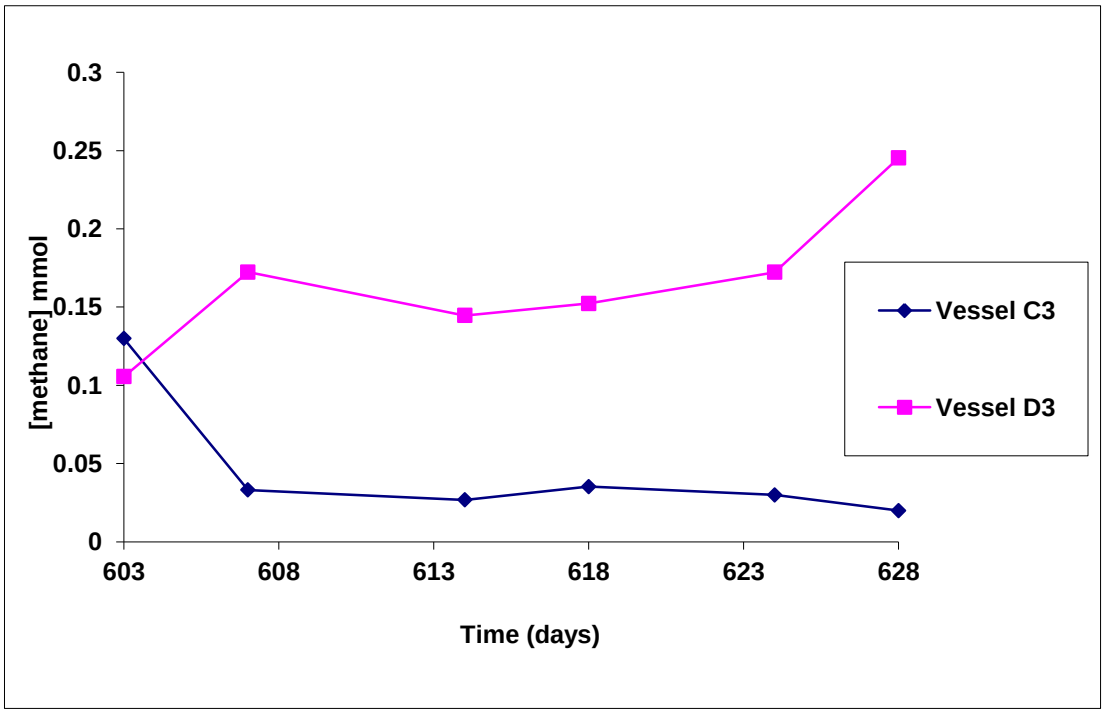


Figure 4.23 Methane against time (days) for vessel C3 (contaminated) and vessel D3 (uncontaminated), contaminates were introduced to Vessel C3 on day 603.

The methane production in vessel C3 has been greatly reduced due to the contamination indicating the inhibition of the methanogenic bacteria (archaea).

Figure 4.24 shows the gel picture of a DGGE gel containing DNA from samples extracted vessels C3 and D3; the lanes of importance are marked on

the image. As shown earlier in this chapter vessel 3 of a system produces the majority of the methane. On analysis of the DGGE gel stain (Figure 4.24), it can be clearly seen that both vessel C3 and D3 contain a number of bacteria and archaeal species.

There appears to be only one bacterial species of a difference between the two vessels D3 and C3, lanes 3 and 4 respectively, and this species does not appear to be one of the predominant ones due to the low intensity of the band (arrow 1). This result indicates the concentration of the contaminants do not appear to have affected the bacterial population.

System C was contaminated with DMP and *o*-cresol, on analysis of the archaeal gel bands for C3 and D3 (lanes 2 and 1 respectively) it can be seen that there are a number of bands present in the lane 1 (vessel D3) which are not present in lane 2 (vessel C3), these bands are highlighted by arrows 2 and 3. The difference in these lanes may be due to the toxic effect of the phenolic compounds. From the gel analysis of the archaeal DNA in vessels C3 and D3, a population change was noted which was caused by the contamination of vessel C3 with the phenolic compounds. While there were still a number of bands, which matched up, and the predominant species appeared to be still present, there were bands missing from lane 2, indicating the loss of some archaeal species. The fact that the predominant bands were still there may indicate that while methane production has diminished in vessel C3, the archaea that produce the methane may only be inhibited and were not necessarily dead, although it must be noted that PCR does not separate live and dead bacteria (Nocker and Camper, 2006), therefore the DNA discovered may also be from dead bacteria. In the lane (lane 2) containing the archaeal DNA from vessel C3, a band was observed which was not found in vessel D3, this band may just be archaeal differences as shown in Figure 4.24 with

the bacterial DNA, or it may be that another species of archaea which would normally be out competed for substrates by the normal archaeal flora, may now be able to thrive due to the inhibition of other species by the phenolic contaminants.

Due to the complex nature and bacterial diversity of the sewage sludge inocula some of the bacterial and archaeal bands seen on the DGGE gel stains may be species not under study for example facultative organisms.

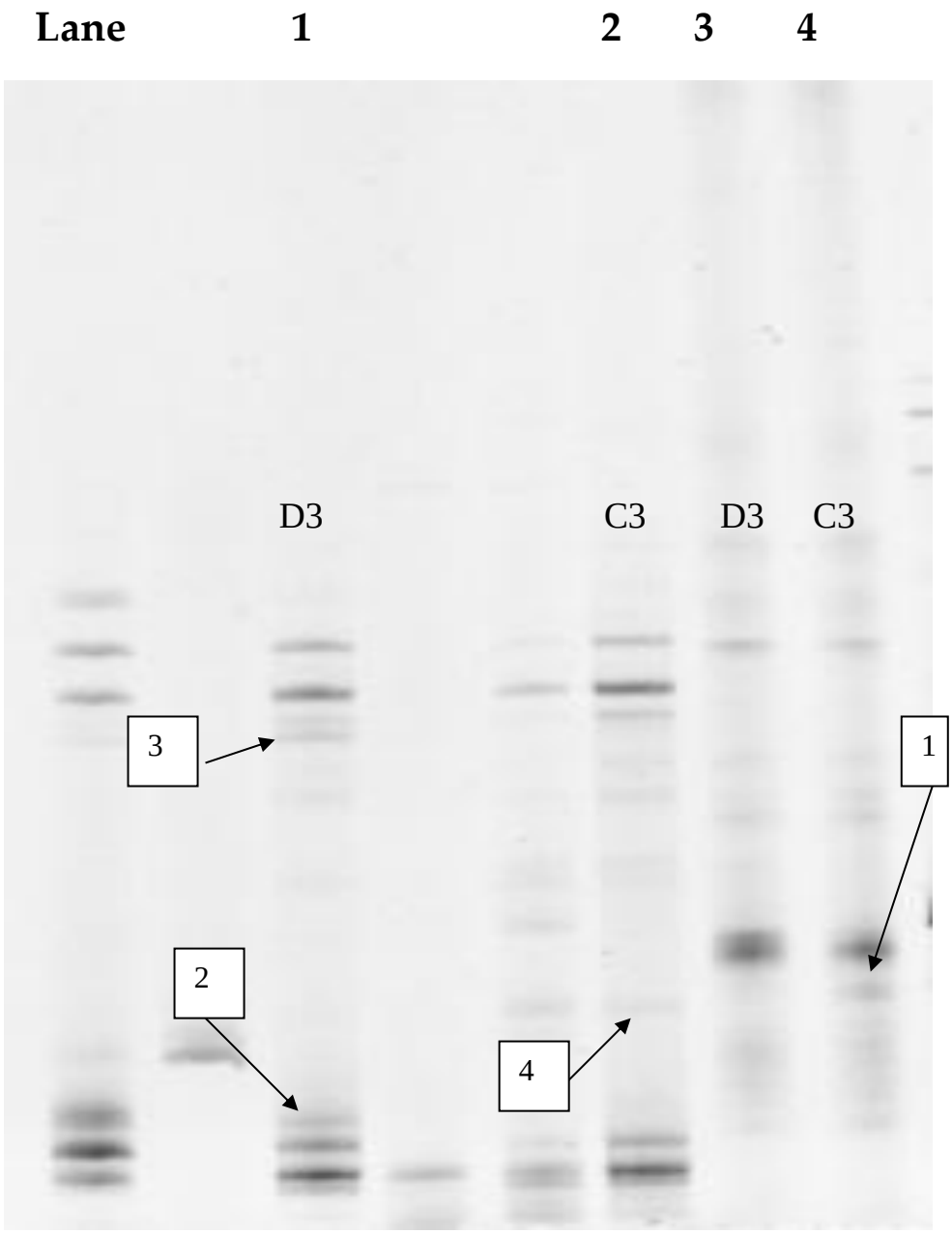


Figure 4.24 DGGE gel shows differences in bacterial populations due to inhibition of bacteria and archaea in vessel C3 (caused by exposure to combined concentrations of DMP and *o*-cresol compared to the populations in the uncontaminated vessel D3. Lane 1 archaeal population of vessel D3, lane 2 archaeal population of vessel C3, lane 3 is the bacterial population of vessel D3 and lane 4 is the bacterial population of vessel C3. Arrows 1 to 4 show population shifts between systems C3 and D3).

4.8 Summary

The results obtained utilizing 3-stage continuous flow systems allowed a better understanding of the inhibitory concentrations exhibited by DMP and *o*-cresol on the bacterial groups of interest.

Results obtained from the batch culture experiments in Chapter 3 showed that both DMP and o-cresol inhibited methane production. It was however unclear if this was due to inhibition of acetoclastic methanogens, hydrogenotrophic methanogens or inhibition of other bacterial groups within the consortium, which are essential for to produce the pre-cursors and redox conditions for methanogenesis. By using the three stage systems, bacterial groups were separated into different vessels, although this was not complete segregation and overlaps in bacterial activity could be seen across the different vessels of the systems. None the less the predominant bacterial processes in each vessels could be observed (Figure 1.5). On analysis of stabilisation results (Figures 4.3, 4.4, 4.9 and 4.10) it was reasoned that the methane producing archaea had been separated into two groups, acetoclastic (vessels 3) and hydrogenotrophic (vessels 2).

The results from vessels 2 and 3 of both systems showed that it took the same concentrations of the pollutants to completely inhibit methane production in both of these vessels. It was noted that the lower concentrations of DMP and *o*-cresol exhibit more of an effect on the hydrogenotrophic bacteria in vessels A2 and B2, than the acetoclastic methanogens in A3 and B3. This indicates that the hydrogenotrophic methanogens were more susceptible to lower concentrations of the phenolic compounds, although these concentrations did not cause complete inhibition of this bacterial group. Due to the nature of

the systems and the fact that contamination started from the bottom vessel up, the knock on effect of inhibition of other bacterial groups, which play an important role in creating substrates and conditions for methanogenisis, can be dismissed. Therefore the results showing inhibition of methane production were due to direct inhibition of methanogenic bacteria.

The results from this study show that DMP and *o*-cresol inhibited sulphate reduction more than they inhibited any of the other bacterial process analysed; complete inhibition was noted at concentrations of 1.2mM DMP and 3.7mM *o*-cresol compared to 2.5mM DMP and 4.6mM *o*-cresol for methanogens. These results expand on those found in Chapter 3 and indicate that the compounds DMP and *o*-cresol exhibit a greater toxic effect on SRB than any of the other bacteria studied, this result was also noted by Sulisti (1994). In the present experiment the sulphate reducing bacteria were already an established community in the top vessels of the systems before the addition of o-cresol and DMP, this makes the results between the bacterial groups in different vessels more comparable. Although the inhibition of sulphate reduction may be an indirect effect, unlike the methanogenic bacteria, which were in vessels 2 and 3, the sulphate reducing bacteria were predominant in vessel 1. Vessel 1 was the top of both systems and where the input media entered the system. Therefore inhibition of the fermentative and acidogenic bacteria, which produce VFA from the metabolism of cellobiose, may cause sulphate reduction to be reduced due to lack of substrate. A solution to this would be to use a substrate utilised directly by the sulphate reducing bacteria therefore any inhibition noted would be from a direct effect.

The concentrations of DMP and *o*-cresol found in this study to cause complete inhibition of sulphate reduction and methane production were lower than the concentrations needed to obtain the same effect in the batch culture experiments of Chapter 3. In the case of DMP, the concentration needed to cause complete inhibition of sulphate reduction in the batch cultures was 3.7mM, whereas only 1.2mM was needed in system A to obtain the same effect. A concentration of 2.5mM DMP caused complete inhibition of both methanogenic groups in system A, even at the highest concentration of DMP (4.1mM) methanogenisis was not completely inhibited in the batch cultures. *O*-cresol had an increased inhibitory effect on individual bacterial groups in system B compared with the same groups that were present in the batch cultures utilised in Chapter 3. A concentration of 3.7mM o-cresol caused complete inhibition of SRB in system B. To get the same effect in the batch cultures 7.4mM *o*-cresol was needed. Almost complete inhibition of methanogenisis was recorded in the batch cultures at concentrations of *o*-cresol ≥ 9.3mM; complete inhibition of the same bacterial groups in system B was observed at 4.6mM *o*-cresol.

It therefore appears that the bacterial groups are offered more protection against DMP and *o*-cresol within a batch culture than when the groups are separated as they were in a system. A possible explanation for this is that in the batch culture there were more particulates present in the form of the sludge inocula. These particulates could offer the bacteria in the batch cultures some protection and allow them to form biofilms. In the systems the bacteria were separated into their individual groups, thus decreasing the tolerance they have to DMP and *o*-cresol. Due to the systems having a

continual flow the bacteria in the vessels are continually challenged making them more susceptible to the toxic effects of the phenols.

The multi stage systems allowed the analysis of the individual groups of bacteria and created a more detailed picture of the effects DMP and *o*-cresol exhibited on these groups. As with the batch cultures, the system results showed DMP too be more toxic than *o*-cresol.

In the groundwater environment the bacteria are present in the aquifers, and it appears that they are spatial separated due to nutrient plumes and redox conditions (Franklin *et al.*, 2000), therefore they may not be offered the same protection they would be given when they are altogether. This would mean that the lower concentrations of contaminants might have a greater effect on the bacteria found in groundwater.

Multi-stage systems allow for a more detailed analysis of the effects of toxic compounds and show how bacterial groups can be separated, but must coexist with overlapping activity domains.

Analysis of the molecular results obtained from this experiment showed population differences between vessels of the same system (Figure 4.22) and also differences in populations between systems C and D were observed (Figure 4.24). Slight differences were noted between system populations before contamination, although both systems were run under identical conditions. The differences may be due to the unpredictable nature of biological systems and the fact that it cannot be guaranteed that the inocula for each system contained identical microbes, even though it was from the

same sample container. Even though slight differences in bacterial and archaeal populations were noted, the predominant species in the vessels of system C were found in the corresponding vessels of system D. Due to these differences between the control system D and the contaminated system C exact comparisons of population shifts due to contamination cannot be obtained.

The DGGE gel picture obtained from the extracted DNA from vessel C3 after contamination with *o*-cresol and DMP, compared to the DNA from the control system showed differences in bacterial and archaeal populations. The DNA was extracted after the methane level in vessel C3 had decreased; therefore it was known that the methanogenic archaea had been inhibited. This would indicate that the population shifts could be due to the toxicity exhibited by the contaminants.

Although differences were noted in the populations of the vessels, the predominant species appeared to remain the same. It has been previously stated Section 1.7 that there are a number of problems associated with PCR DGGE. PCR does not discriminate between DNA from live or dead bacteria (Dooms et al., 2007) and DNA can persist in the environment up to three weeks after cell death (Nocker and Camper 2006). Therefore in this experiment the predominant bands found on the DGGE gel from system C after contamination could have been DNA amplified from dead bacteria and archaea.

The use of DGGE coupled with analytical techniques enabled further understanding in to the activity and population differences between systems and vessels, and the effect contaminants exhibited on the bacterial

populations of the multi-stage systems. Ideally if time had permitted further work would have involved the sequencing of the DNA bands of interest extracted from the DGGE gels, which would have enabled the naming of the different types of bacteria. This would then allow further study into the effects *o*-cresol and DMP have on these bacterial populations with the possibility of discovering a bacteria or archaea which were resilient to these chemicals, in turn enabling further work into the biodegradation of these compounds.

Chapter 5

Combined toxicity of *o*-cresol and 2,4-dimethylphenol

5.1 Introduction

The previous experiments in this study focused on the inhibition of the function of anaerobic bacteria by the phenolic compounds DMP and *o*-cresol individually. Such compounds are found in crude oil, and used in many industrial and pharmaceutical processes (Kirbet *et al.*, 2000; Abd-El-Haleem *et al.*, 2002; Acuña-Argüelles et *al.*, 2003) and are known to readily enter the groundwater environment (Godsy *et al*., 1983; Thiergartner, 2006). Chapter 3 gave an indication of the concentrations required for each of these compounds to exhibit an inhibitory effect on a number of bacterial processes; sulphate reduction, acidogenic and acetogenic processes and methanogenesis. In the groundwater environment it is unlikely that DMP or *o*-cresol would be found individually since they are usually found in a mixture of chemicals. The present study was therefore set-up to observe the combined inhibitory effects when both DMP and *o*-cresol were added to a mixed anaerobic bacterial consortium. From the analysis of the two compounds, the threat of multiple compounds may be ascertained.

Previous studies have been conducted on the toxic effects of individual phenolic compounds on anaerobic bacterial associations (Smolenski and Sulfita, 1986; Sulisti *et al*., 1997; Spence *et al*., 2001; Acuña-Argüelles et *al*., 2003). While these studies gave a good indication of the toxic concentrations of the individual compounds, they are rarely environmentally relevant. Environmental contaminants normally contain many toxic compounds which readily enter the groundwater environment, therefore there will be many chemicals present that could exhibit toxic effects on the bacteria. These chemicals may interact with each other causing toxic effects that may not be

expected if based solely on the analysis of results from studies that focus on the effects of the individual compounds. It was therefore imperative that the analysis of the combined toxicity of environmental pollutants was studied.

As previously discussed in Section 1.4, there are three possible outcomes when mixed pollutants are added to bacterial cultures, these are:

1. antagonistic effects- if this occurs the effects of the individual chemicals may cancel each other out, thus causing less of an inhibitory effect
2. additive effects- the combined effect of the two chemicals is the sum of the effect of each individual chemical
3. synergistic effect- is a response greater than the sum of the combined effects of the chemicals.

As mentioned in Section 1.4, 2,4-Dimethylphenol and *o*-cresol are both short-chained alkylphenols and classed as polar narcotics. They have a similar structure, with DMP having an additional methyl group attached to the 4th carbon of the aromatic ring. The addition of the methyl group to DMP ring structure decreases its solubility and from the previous studies in Chapters 3 and 4 increases the toxicity of the compound. Both compounds will disrupt the cell membrane of the bacteria changing its structure resulting in cell death (Section 1.4). The effect on the toxicity of the two compounds when they are combined should be theoretically an additive one due to the similarities between them. According to Lin *et al.,* (2004) the joint effects of polar narcotics is a concentration addition i.e. polar narcotics have an additional effect when combined.

In this experiment the compounds will be added together to batch cultures at different concentrations, with the expected result of the combinations to have an additive effect. The toxicity exhibited by the compounds is also dependant on that number and type of bacteria present in the cultures.

5.2 Experimental method

5.2.1 Sulphate

Batch culture bottles, in triplicate, were set up as described in Section 3.2.1 Control cultures with no *o*-cresol or DMP added were set-up alongside cultures containing concentrations of *o*-cresol and DMP (Table 5.2), and cultures containing mixed concentrations of the two compounds. All cultures were supplemented with SO_4 (4.2mM) and stored at 30°C in darkness to make sure photo bacteria were not utilising the carbon source. Analysis of sulphate was on days 1 and 14 using ion chromatography (Section 2.2.2). The experiment ended when all sulphate had been completely reduced in the control cultures. Sulphate concentrations for the batch cultures were determined using ion chromatography (Section 2.2.2).

5.2.2 Methane

Batch cultures for the analysis of the combined toxicity of DMP and *o*-cresol on methane production were set up as above, with the exclusion of the two toxic compounds which were added to the cultures after the methanogenic bacteria had established themselves, after 30 days; the concentrations added are shown in Table 5.2. To ensure methane production was not affected by the inhibition of other bacterial groups which provide the substrates (e.g. acetate) utilised by the methanogenic bacteria, additional acetate (20mM)

was added to the cultures prior to them being perturbed by the alkylphenols. This supplementation of acetate enabled the direct analysis of the effects of the two compounds on methanogenic bacteria by increasing the substrate utilised by these bacteria, therefore allowing the effects of the compounds on acetate producing bacteria to be ignored. The amount of sodium acetate ensured that there was an abundance of acetate for methane production. The experiment was ended after 71 days from batch culture set up, it was determined that at this point the methane production rate was ceasing in the control batch cultures when the methane rates decreased. In hindsight the experiment should possibly been stopped when the acetate levels in the control cultures reached zero. Analysis of methane production followed the protocol set out in method section 2.2.3.

5.2.3 Percentage inhibition concentrations

The percentage inhibition concentrations (Figures 5.1 and 5.2) used in these experiments were determined from analysis of the sulphate concentrations of cultures perturbed with DMP and *o*-cresol (Sections 3.3.1 and 3.4.1). The % inhibition concentrations were achieved by calculating the percentage of sulphate left at the end of the experiment (using the start and end concentrations) and subtracting it from 100. From the % inhibition charts (Figures 5.1 and 5.2; and Table 5.1) the concentrations of DMP and *o*-cresol that caused 20%, 40%, 60% and 80% inhibition were determined by extrapolation of the points on the charts. An assumption was made that since both *o*-cresol and DMP are similar in structure and function, then the addition of these two toxic compounds should have an additive effect on the

inhibition of the bacteria, therefore theoretical additive inhibitory concentrations were calculated (Table 5.2).

After completing this experiment and on review of the results for the thesis, it was discovered that a more statically sound method for finding inhibition concentrations could have been used. It was decided that even though the data was not used to obtain % inhibition concentrations, it should still be included in this thesis.

Probit dose response analysis using xlstat software (Addinsoft; Paris, France) was carried out on the sulphate reduction and the methane production results for the batch sets contaminated with *o*-cresol and DMP (Chapter 3). This analysis gave the probable concentrations that would cause a % inhibition and from it the IC_{50} (inhibition concentration 50%) could be obtained. These results can be found in appendix III.

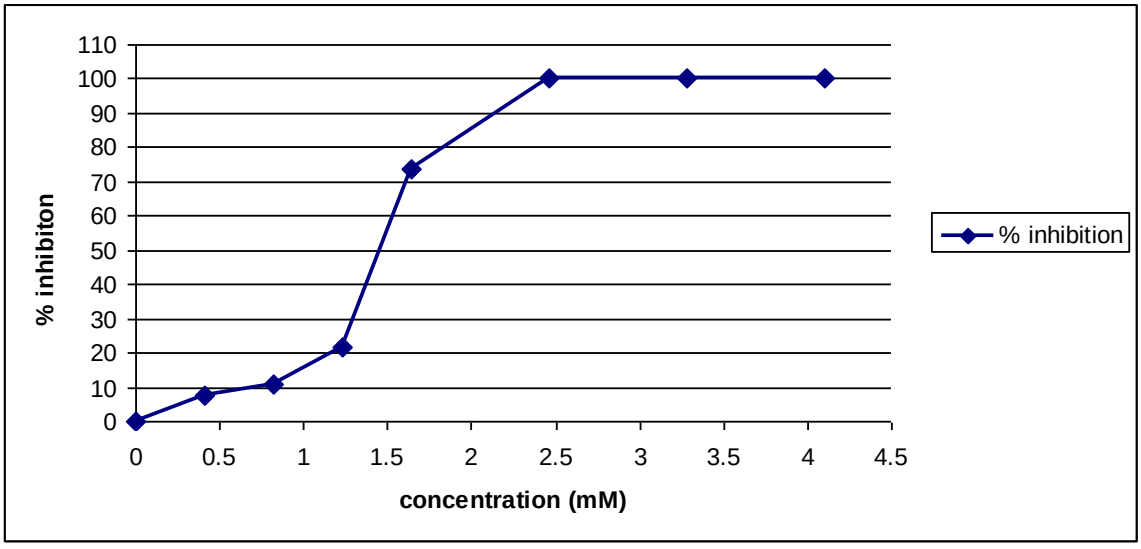


Figure 5.1 % inhibition of sulphate reduction versus 2,4-dimethylphenol concentration

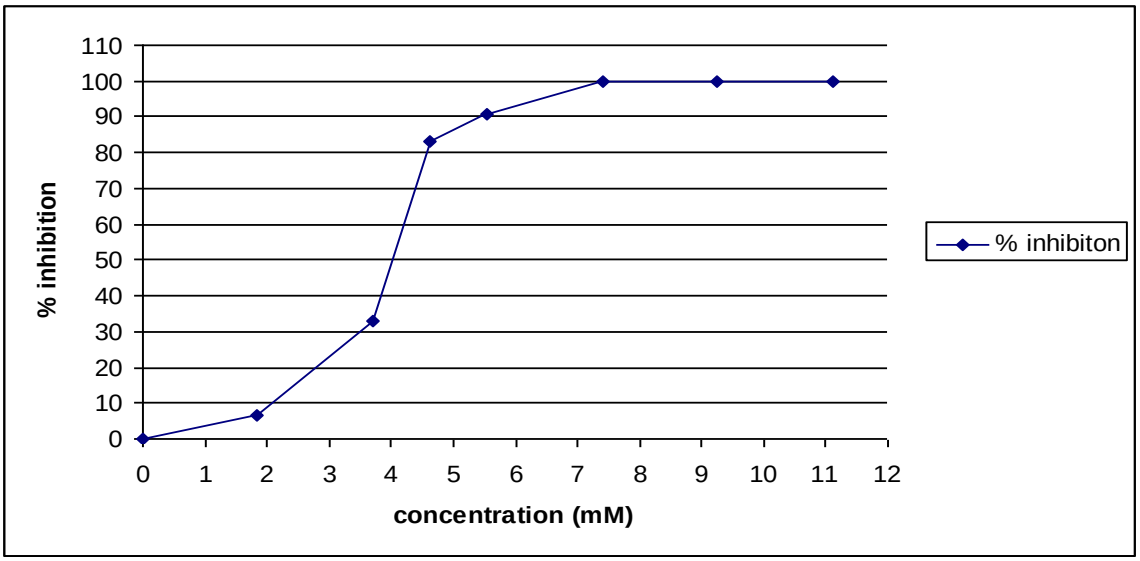


Figure 5.2 % inhibition of sulphate reduction versus *o*-cresol concentration

Table 5.1 % inhibition for o-cresol and DMP determined from Figures 5.1 and 5.2

% inhibition	[*o*-cresol] mM	[2,4-DMP] mM
20	2.80	1.15
40	3.80	1.39
60	4.25	1.53
80	4.50	1.81

Table 5.2 Concentrations of DMP and o-cresol required to give combined theoretical % inhibition

Total theoretical % inhibition	% inhibition DMP	% inhibition *o*-cresol	[DMP] mM	[*o*-cresol] mM
60	0	60	0	4.25
60	60	0	1.53	0
60	40	20	1.39	2.80
60	20	40	1.15	3.80
80	0	80	0	4.50
80	80	0	1.81	0
80	40	40	1.39	3.80
80	60	20	1.53	2.80
80	20	60	1.15	4.25

5.3 Results and discussion

5.3.1 Sulphate

The experiment was completed after 14 days when it was clear that all sulphate had been reduced in the control cultures, which contained no contaminants. The control cultures therefore had no inhibitory effect from *o*-cresol and DMP, thus resulting in 0% inhibition for sulphate reduction. Figures 5.3 and 5.4 show the comparison of the % inhibition for both control and contaminated cultures. The data from the experiment can be found in Appendix III.

5.3.1.1 60% inhibition

Figure 5.3 clearly shows that in this culture set, 4.25mM *o*-cresol and 1.53mM DMP both have an inhibitory effect on sulphate reduction. The theoretical % inhibition for these concentrations is 60%, sulphate reduction is decreased by 64% for both compounds, an acceptable result since the complex nature of biological systems means that it would be impossible to replicate previous experiments exactly, due to population shifts and bacterial numbers in inocula.

The inhibition of sulphate reduction in the batch cultures sets perturbed with the mixtures of *o*-cresol and DMP, containing either 2.8mM (20%) *o*-cresol/ 1.39mM (40%) DMP or 3.8mM (40%) *o*-cresol/ 1.15mM (20%) DMP, was calculated to give a theoretical inhibition of 60% (Table 5.2). This assumed that the combined effect of these compounds would be additive. The actual percentage of sulphate inhibition as a result of combining *o*-cresol and DMP for both mixtures was approximately 90% for both mixtures. These results

show that rather than having an additive effect, the combination of the compounds resulted in a synergistic effect on the inhibition of sulphate reduction.

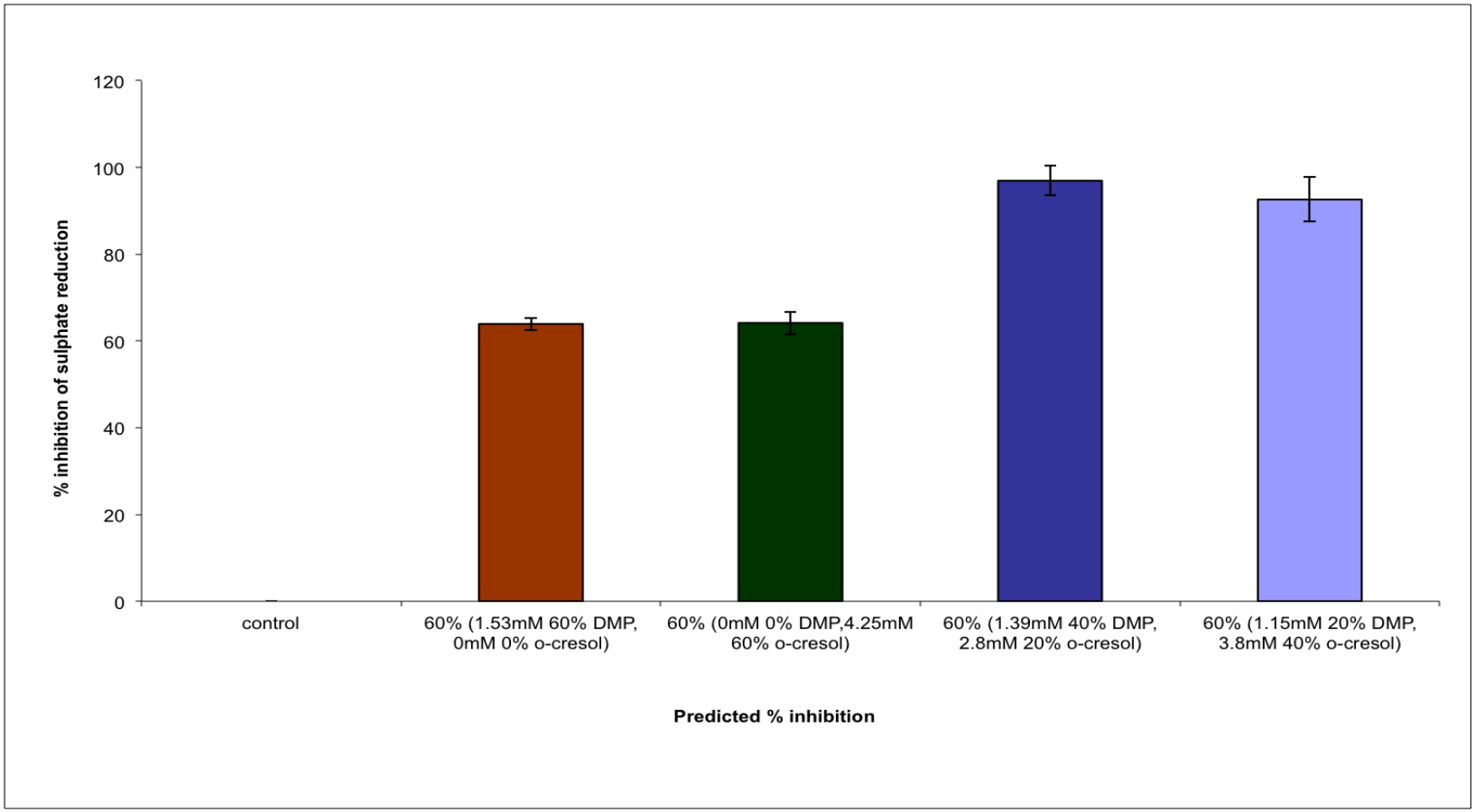


Figure 5.3 Predicted 60% inhibition of sulphate reduction for the combined toxicity of o-cresol and DMP. Calculated using Table 5.2. (Error bars are the standard deviations of the triplicate cultures, these can be found in Appendix III).

5.3.1.2 80% inhibition

Batch cultures were set up containing individual concentrations of DMP and *o*-cresol, which were known to inhibit sulphate reduction by approximately 80%. These concentrations were 1.81mM DMP and 4.5mM *o*-cresol and in this study they inhibited sulphate reduction by approximately 70%. This once again shows the diverse nature of working with bacteria, slight changes in the inocula may have caused the results to differ from the first experiments (Figure 5.4). It was unlikely that these concentrations would inhibit this process by exactly 80% since there are differences in the microbial systems as a consequence of the seasonal changes in the bacterial content and microbial activity of the inocula as previously mentioned in Section 3.4.3.

Three different cultures sets containing mixtures of DMP and *o*-cresol with concentrations adding up to a theoretical inhibition value of 80% were also set up. The concentrations of each compound in these cultures were: 3.8mM (40%) o-cresol/ 1.39mM (40%) DMP, 4.25mM (60%) *o*-cresol/ 1.15mM (20%) DMP and 2.8mM (20%) *o*-cresol/ 1.53mM (60%) DMP respectively.

From the results in Figure 5.4 it can be seen that each of these mixtures appears to exhibit a synergistic effect on sulphate reduction. The combination of 3.8mM *o*-cresol and 1.39mM DMP caused a 91% inhibition; 4.25mM *o*-cresol and 1.15mM DMP resulted in an inhibition of 99%; and 2.8mM *o*-cresol/ 1.53mM DMP inhibited sulphate reduction by 96%.

The results obtained in the combined effects study indicate that DMP and *o*-cresol when combined exhibit a synergistic effect, greater than the sum of the individual effects of both compounds, which results in a greater inhibitory effect on sulphate reduction.

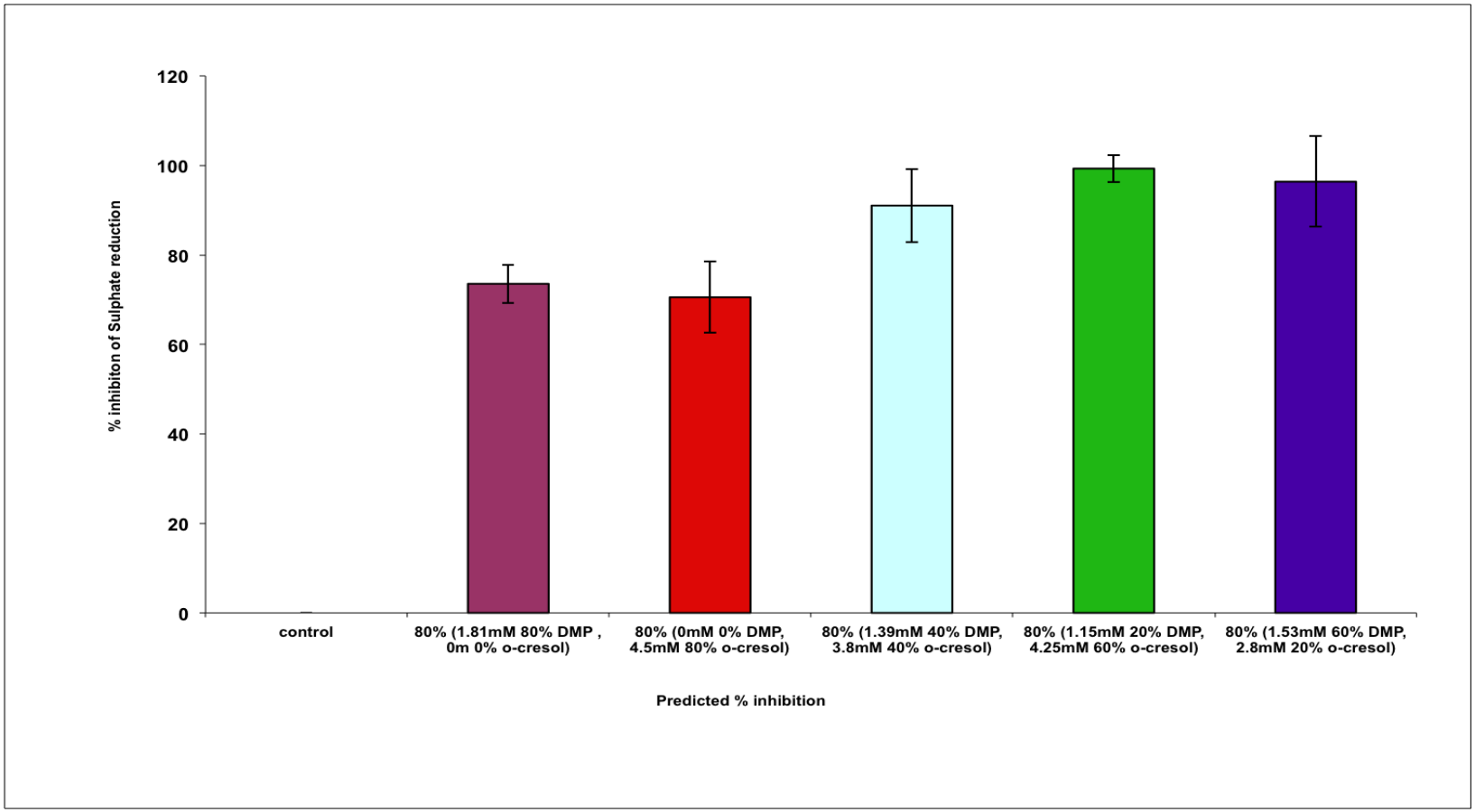


Figure 5.4 Predicted 80% inhibition of sulphate reduction for the combined toxicity of o-cresol and DMP. Calculated using Table 5.2. (Error bars are the standard deviations of the triplicate cultures, these can be found in Appendix III).

5.3.2 Methane

Batch culture sets were set-up to examine the combined effects of *o*-cresol and DMP on bacterial methane production (Section 5.2.2). Contamination of the cultures was initiated after all the methanogenisis was established.

Figures 5.5 and 5.6 show the percentage inhibition of methane production for batch culture sets perturbed with DMP or *o*-cresol and both of these compounds combined when compared to control cultures. The concentrations added were the same as those used in the analysis of sulphate reducing bacteria Table 5.2. Although these concentrations were known not to cause the same amount of inhibition to methane production as they did to sulphate reduction, they were used to give a further comparison of the effect of these compounds on both bacterial groups. On analysis of Figures 5.5 and 5.6 it can be seen that the % inhibition of methane production is much less than the % inhibition of sulphate reduction (Figures 5.3 and 5.4) for the same concentrations of contaminants, giving a further indication that the sulphate reducing process is more susceptible to the toxic effects of these compounds than the methane production.

5.3.2.1 60% inhibition

Figure 5.5 shows the % inhibition for methane production of batch culture sets contaminated with *o*-cresol or DMP and those contaminated with both of these compounds. The concentrations of contaminants were the same as those used in the sulphate concentration study Section 5.2.1.

The concentrations of the individual contaminants that caused approximately 60% inhibition for sulphate reduction were DMP- 1.53mM

and *o*-cresol- 4.25mM, but these concentrations only inhibited methane production by 8% and 13% respectively. These results show that the methane producing bacteria are far more resilient than the sulphate reducing bacteria and as there were excess substrates provided for methanogenisis, the results show the effect on the methane producing bacterial population.

Figure 5.5 also shows the percentage inhibition for the combined toxicity of *o*-cresol and DMP for methane production, note these combined concentrations were the same as the theoretical additive inhibition concentrations used against sulphate reduction. The percentage results obtained show that for combinations (2.8mM *o*-cresol and 1.39mM DMP) and (3.8mM *o*-cresol and 1.15mM DMP) of *o*-cresol and DMP the percentage inhibitions are approximately 38%. These results show that the combination of the two compounds appears to produces a synergistic rather than additive inhibitory effect on methane production, with a higher than expected toxic effect on the methanogenic bacteria by comparison with the inhibitory effects of the individual contaminants.

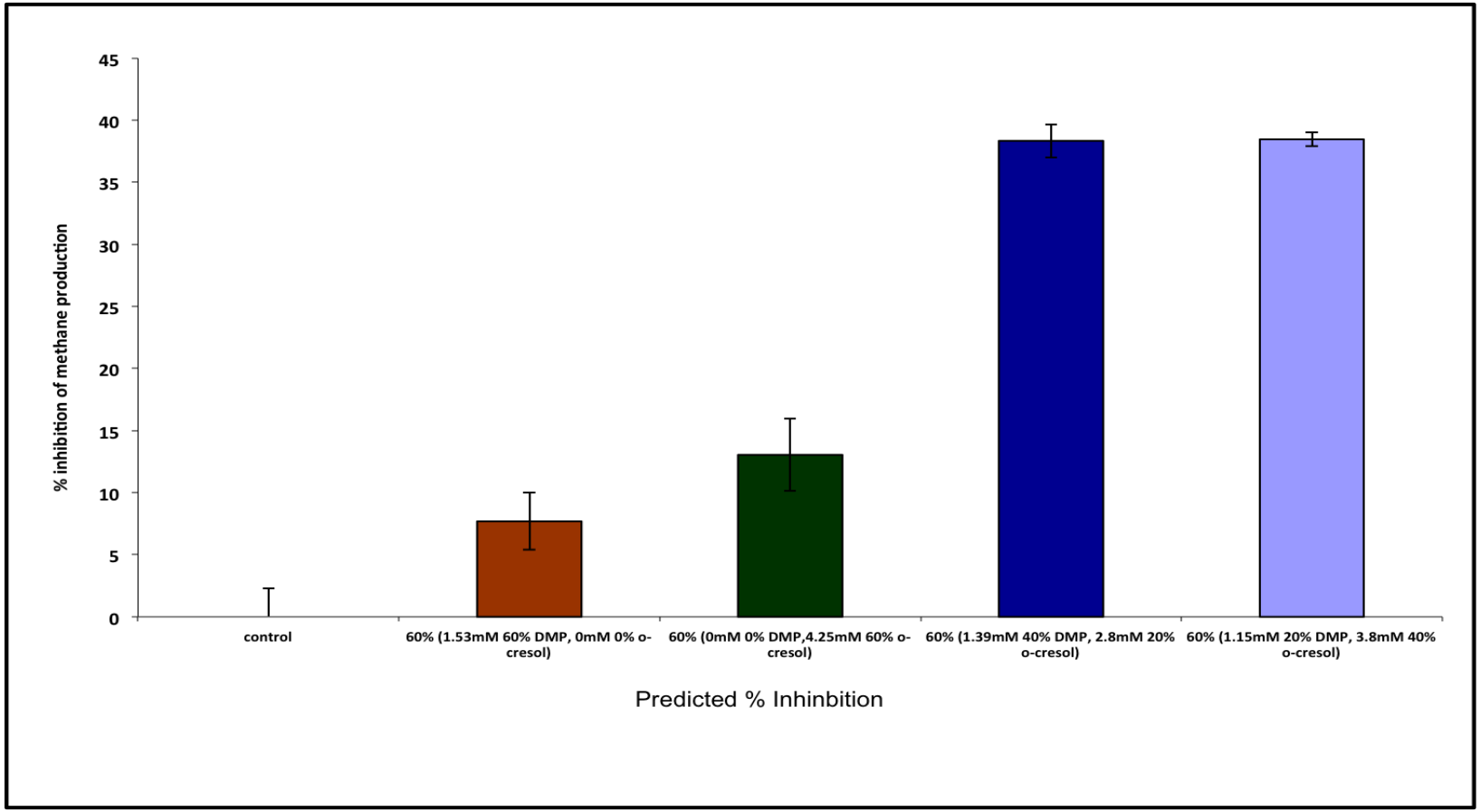


Figure 5.5 % inhibition of methane production for the combined toxicity of o-cresol and DMP. Calculated using Table 5.2. (Error bars are the standard deviations of the triplicate cultures, these can be found in Appendix III)

5.3.2.2 80% inhibition

Figure 5.6 shows the percentage inhibition of methane production after the addition DMP and *o*-cresol, individually and combined, which exhibited a theoretical additive effect of 80% on sulphate reducing bacteria.

The percentage inhibitions caused by the addition of 4.5mM *o*-cresol or 1.81mM DMP were 22% and 23%. Both concentrations caused approximately 70% inhibition of sulphate reduction again showing a higher tolerance of these compounds by methanogenic bacteria c.f. sulphate reducing bacteria.

The percentage inhibition of methane production for the combined concentrations of DMP and *o*-cresol of 3.8mM *o*-cresol and 1.39mM DMP, 4.25mM *o*-cresol and 1.15mM DMP and 2.8mM *o*-cresol and 1.53mM DMP were 42%, 41% and 47% respectively. These results show a far greater effect than the sum of the combined concentrations indicating a possible synergistic effect.

It can be clearly seen that for both experiments into the inhibition of methane using combined pollutants that the toxic effect exhibited on methane production is greater than an additive effect therefore when both compounds are combined they appear to give a synergistic toxic effect on the methanogenic organisms of the cultures. It should be noted that the concentrations of DMP and *o*-cresol used were taken from the percentage inhibition of sulphate reduction; therefore, further work needs to be carried out to assess the synergistic effects of these compounds on methanogenisis.

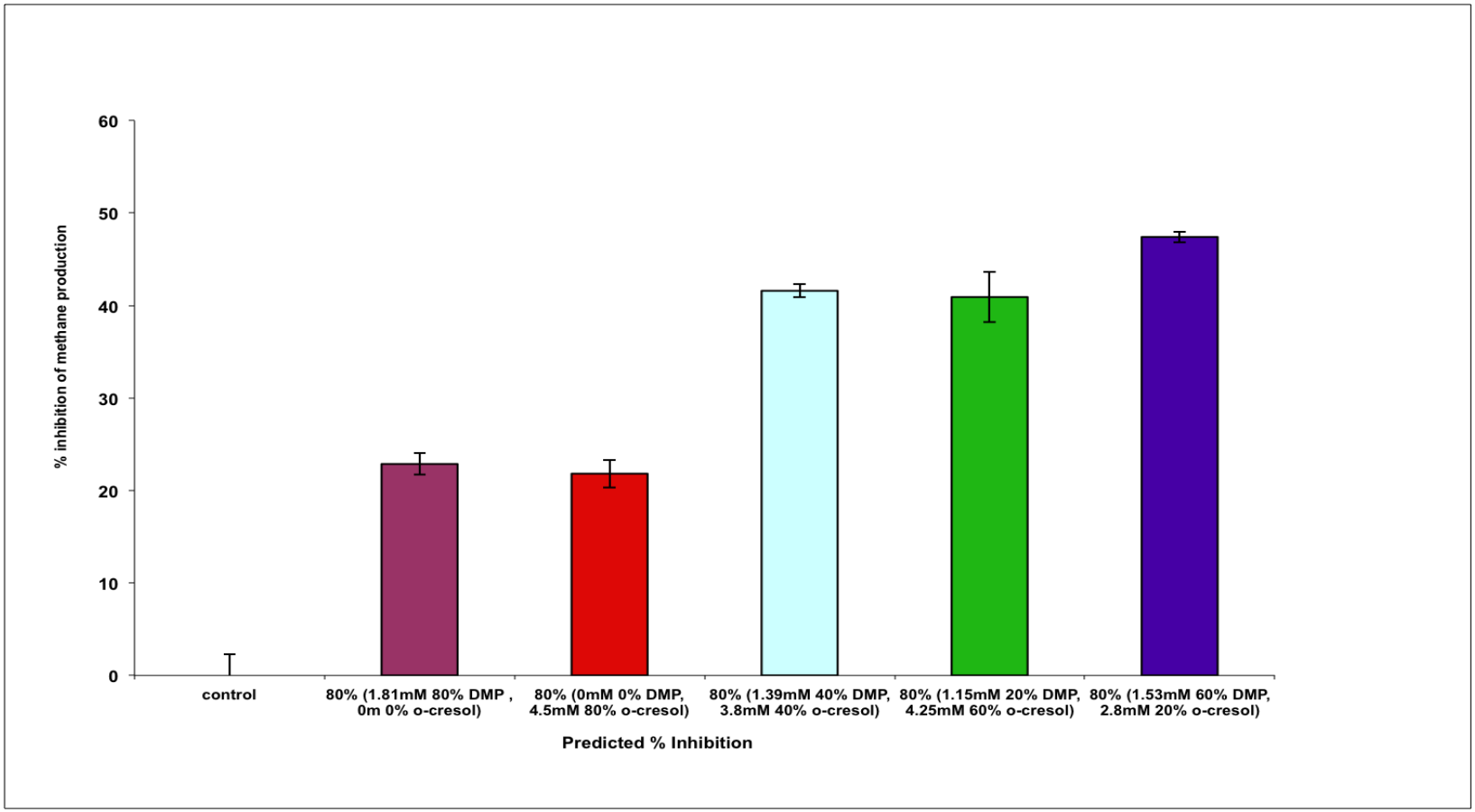


Figure 5.6 % inhibition of methane production for the combined toxicity of o-cresol and DMP. Calculated using Table 5.2. (Error bars are the standard deviations of the triplicate cultures, these can be found in Appendix III).

The percentage inhibition for methane production, caused by the individual concentrations of *o*-cresol and DMP in this experiment, appears to be less than the effect the chemicals exhibited on methane production in Chapter 3. The percentage inhibition of methane production due to *o*-cresol and DMP contaminations at the beginning of the batch culture experiments (Chapter 3) is greater than the percentage inhibition of methane production if these compounds perturbed the cultures after the onset of methanogenisis. Figure 5.7 shows the difference in percentage inhibition of methane production by *o*-cresol or DMP when added before or after the onset of methanogenisis. Although the concentrations of the compounds differ, the difference in inhibition can be clearly seen. These results are similar to those obtained by Sulisti (1994) in a study using refuse as an inocula and *o*-cresol as a contaminant. That study showed that the effect on methane production was greater when the *o*-cresol was added at the beginning of the study i.e. as it was in Chapter 3 of the current study. There are various reasons for the results shown on Figure 5.7:

(i) In this experiment the methanogens were allowed to establish before contamination, which allowed the development of biofilms. According to Brumolle *et al.,* (2014), biofilms over the bacteria far more protection than if they were planktonic cells.

(ii) In the studies conducted in Chapter 3, the cultures were perturbed with the contaminants at the beginning of the experiments. This allowed the study of the overall toxic effects of the contaminants on bacterial consortia, even before methanogenisis was established. As previously mentioned anaerobic consortia have synotrophic relationships (Section 1.3). Therefore due to the contaminants having an affect on other bacterial groups, which produced

pre-cursors for methanogenisis, methanogenisis was affected due to lack of substrate and correct conditions

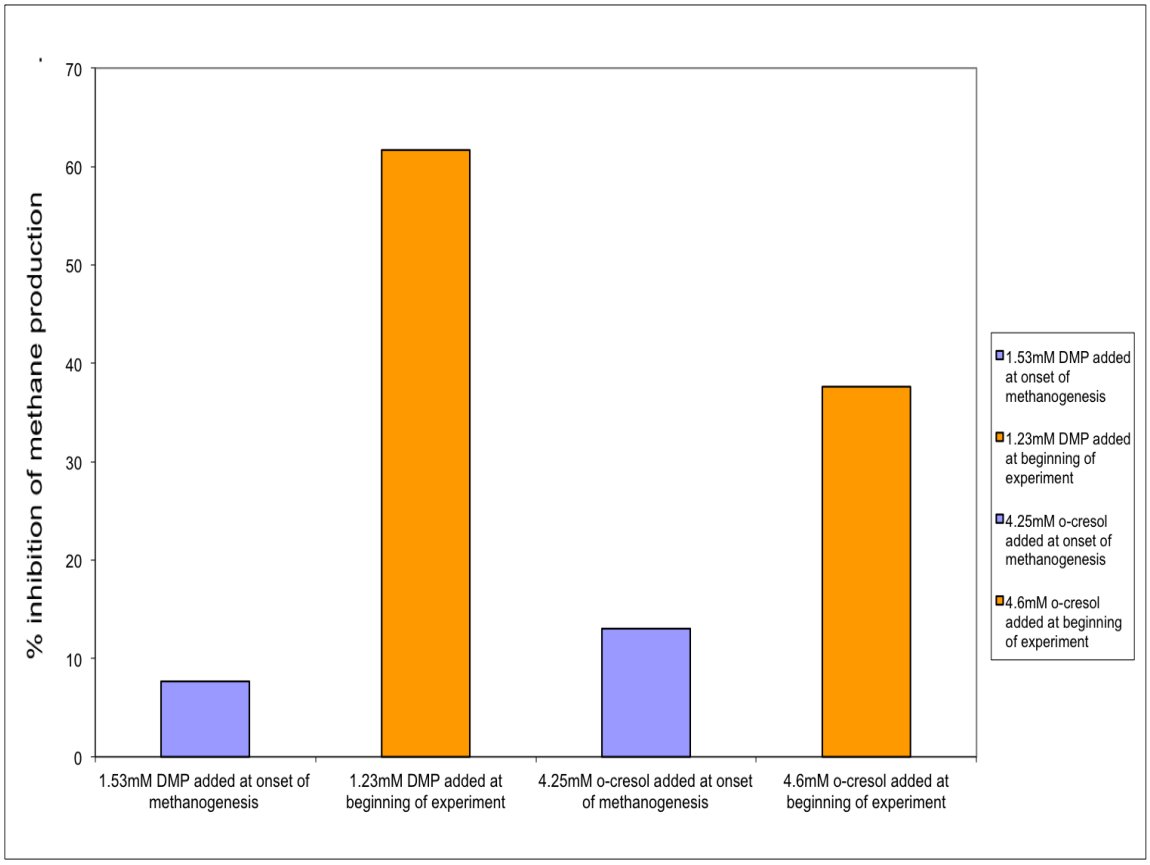


Figure 5.7 Comparisons of individual compound toxicity added at start of experiment or after methanogenisis commenced.

The blue columns are the cultures contaminated after the onset of methanogenisis and the orange columns are those contaminated at the beginning of the batch culture experiments.

5.4 Discussion

5.4.1 Inhibition differences between SRB and methanogens by o-cresol and DMP

From the results in this chapter it can be seen that sulphate reduction is more susceptible to the two phenolic compounds than the methane production (Figures 5.3-5.6). These results have been observed in a previous study (Holmes *et al.,* 2002). Since all the results in the present study showed methanogenic bacteria have more tolerance to the toxicity of *o*-cresol, 2,4-dimethylphenol and both chemicals combined, it can be concluded that the methanogenic bacteria are more robust in the presence of these two phenolic compounds than SRB.

It has been documented that methanogenic bacteria form biofilms (Sulisti, 1994, Brumolle *et al.,* 2014) and biofilms offer cells more protection than when they are in their planktonic state. Hence, these biofilms could be the reason that methanogens are more tolerant to *o*-cresol and 2,4-dimethylphenol.

Methanogenic bacteria are archaea and are also known to be extremophiles (Ciaramella *et al*., 2005). Archaea are known to have different lipid structure in their plasma membrane, which may help in their habitation of extreme environments and makes the archaea less susceptible to pH and oxidation as a consequence of possessing a more stable membrane (Albers *et al*., 2000). The difference in plasma membrane composition could be the reason why methanogenic bacteria are more tolerant towards *o*-cresol and DMP.

On further review of this chapter and the results of the previous batch experiments (Chapter 3), the experiment might have been planned more effectively. In order to do a direct analysis of the effects of the compounds on

sulphate reducing bacteria, the batch cultures should have been set up like the ones in Section 5.2.2 and this would have allowed the sulphate reducing bacteria to establish. When the sulphate was completely reduced in all cultures the cultures should have been re-supplemented with SO_4 (4.2mM) and suitable VFA (acetate) before the addition of o-cresol and DMP. Doing this would have insured that it was the sulphate reducing bacteria being inhibited and not some other bacterial groups in the consortium that the sulphate reducers rely on.

5.4.2 Contamination of already established methane producing cultures

The results in this chapter also show that if the phenolic compounds are introduced after the establishment of methanogenic communities, and there is an abundance of substrates for methane production, the methane producing bacteria show more tolerance towards the phenolic contaminants than when the contaminants are added pre-methane production, as they were in Chapter 3. These results are in agreement with Sulisti's (1994) study, which showed that methanogenic bacteria were less susceptible to the toxic inhibitory effect of *o*-cresol if the contaminant was added after establishment of methanogenisis. This may be due to population size; there should be a greater number of methanogenic bacteria in the batch cultures where the contaminant is added after the establishment of methanogenesis. The results are due to the fact that there is an abundance of methane substrate (acetate) in the batch cultures studied in this chapter showing that the reduction in methane production noted in Chapter 3 was not solely down to inhibition of methanogenic bacteria, but also to the bacterial groups that provide the pre-cursors for methanogenisis such as acetogenic bacteria. These results indicate that if these pollutants entered the environment, the knock-on effect on the

bacterial consortia would disrupt the anaerobic process as a whole, since each individual bacterial group depends on the other groups to maintain the optimum environment (as discussed in Section 1.3)

5.4.3 Synergism in both SRB and Methane

The effect that was expected from the combination of the chemicals was an additive effect, due to the chemicals being similar, rather than the synergistic effect, which was noted. As previously mentioned in Section 1.5 *o*-cresol and DMP are polar narcotics, therefore it was assumed that their combined effect would be a concentration addition. No literature could be found on synergistic effect of two polar narcotic compounds.

As mentioned in Section 5.2, on completion of the experiment probit dose response statistics were run on the data from Chapter 3. This data yielded more statistically correct inhibition concentrations. Table 5.3 shows a the data obtained from probit analysis of the sulphate reducing experiments in chapter 3 and the data calculated according to Figures 5.1 and 5.2

Table 5.3 Comparison of %inhibition and probit analysis data.

% inhibition	[*o*-cresol] mM From fig. 5.2	[*o*-cresol] mM (Probit)	[2,4-DMP] mM From fig. 5.1	[2,4-DMP] mM (Probit)
20	2.80	3.27	1.15	1.22
40	3.80	3.77	1.39	1.39
60	4.25	4.21	1.53	1.53
80	4.50	4.77	1.81	1.70

It was thought that the data calculated from Figures 5.1 and 5.2 may not have been accurate and that the inhibition concentrations may have been too high. This was the reason for the probit analysis. On analysis of Table 5.3 it can be seen that there are differences between the probit dose response concentrations and the calculated concentrations. Although the table does show that the combined concentrations used in this experiment were not too high and almost match the statistical data. Analysis of the probit data for inhibition of methane production (Appendix III) shows that inhibition of methane production required a much greater concentration of the phenolic compounds. It may be that the experimental set-up was wrong and there could have been an error in the addition of the chemicals. Due to the time restraints of the study repeat analysis, which could have backed up the results or shown any error, was impossible

Therefore the reasons for the synergistic effect by combining DMP and *o*-cresol are unknown and further work needs to be conducted including repeat experiments to ensure that the results are correct.

5.4.4 Environmental impacts of combined pollutants

No literature could be found on the combined effects of *o*-cresol and DMP. Other studies have shown the combined effects of different compounds which have similar structures and exhibit polar narcosis, and that it is easy to predict the additive effects of polar narcotics (Lin *et al.,* 2003, Lin *et al*., 2004). Since some research has shown that these two chemicals are found to enter the environment as part of chemical mixtures (Godsy *et al*., 1983; Thiergartner, 2006)) more work should be undertaken to study the effects of

chemical mixtures on bacterial populations, rather than studies where the effects of the individual chemicals are prioritised.

O-cresol and 2,4-dimethylphenol are two low molecular weight alkylphenols present in many industrial wastes known to readily enter the environment, and, while they may not be the most toxic compounds, even at low concentration, in the mixtures they are found in, they are toxic to bacteria and aquatic life. Due to their low molecular weight they are known to migrate rapidly from their initial source (Guange *et al*., 2000). The results shown in Chapters 3 and 4 indicate that these chemicals are toxic to anaerobic consortia and in this chapter it was discovered that they might have a synergistic inhibitory effect towards the anaerobic bacterial groups in these consortia.

In the environment it would be unlikely that one of these chemicals would be a single pollutant, as they are normally found as part of a chemical consortium, for example in crude oil, therefore as most studies have only concentrated on the inhibitory effects of single compounds, they do not seem environmentally relevant. Ideally a whole consortium of chemicals from an environmental mix would have been investigated. It was reasoned that, since *o*-cresol and DMP were similar, an investigation into them singularly and then combined would shed light on the combined effects these phenolics have on anaerobic growth consortia.

Chapter 6

Final conclusions

6.1 Conclusions

This study was set up to investigate the inhibitory effects that *o*-cresol and 2, 4-dimethylphenol exhibited on anaerobic growth consortiums and to discover the effect a mixture of both these compounds would have on these organisms. These chemicals were chosen since they are polar narcotics, which readily enter the groundwater environment.

The inocula used throughout the study was return activated sludge and the temperature all experiments were carried out at was 30°C. The experiments were used as a model for groundwater bacteria and other anaerobic growth consortiums. In the case of groundwater, the bacterial and archaeal species may differ from the inocula used, but the bacterial groups would be similar, the temperature of all the experiments was set at 30°C to ensure growth rates were not too slow. If the experiment had used the temperatures found in groundwater environments, which are considerably lower than 30°C, the bacterial growth would have been too slow. The increase in temperature may have affected the dominant species in the experiments however, on analysis of the experiments, it was shown that all the expected processes e.g. acidogenesis, actetogenesis, sulphate reduction and methanogenesis were present, therefore the dominant and desired bacterial groups were present.

Previous studies have analysed the effects DMP and *o*-cresol had on anaerobic bacterial associations (Boyd *et al*., 1983; Smolenski and Sulfita, 1987; Sulisti 1994; Spence *et al*., 2001; Holmes *et al.,* 2002). The work carried out by Holmes *et al., (*2002) showed the inhibitory effect of *o*-cresol on the anaerobic bacteria of landfill refuse. In the present study the inhibitory concentrations for *o*-cresol and DMP on anaerobic growth consortiums were

obtained using batch cultures and continuous flow systems inoculated with return activated sludge. Initial experiments utilizing batch cultures were employed to find the inhibitory concentrations and the effects that both compounds exhibited on the sludge inoculum mixed with mineral media.

In the batch culture studies all concentrations of *o*-cresol (1.85mM, 3.7mM. 4.6mM, 5.6mM, 7.4mM 9.3mM and 11mM) and DMP (0.41mM, 0,82mM, 1.23mM, 1.64mM, 2.46mM, 3.28mM and 4.1mM) exhibited some level of inhibition on the bacterial groups analysed.

O-cresol concentrations ≥ 1.85mM exhibited an inhibitory effect on all the bacterial groups analysed, although this may have been indirect due to synotrophic nature of the anaerobic consortia (Section 1.3). Therefore the inhibition of bacterial processes may have been due to the inhibition of other species in the consortium i.e. inhibition of acetogens affects methane production due to lower levels of acetate production. At concentrations ≥ 7.4mM complete inhibition of sulphate reduction was noted and the acetogenic and methanogenic processes were greatly reduced, concentrations ≥ 9.3mM appear to cause complete inhibition of methane production.

A similar trend was observed in the DMP batch experiment, with all concentrations exhibiting inhibitory effects on the bacterial groups (concentrations ≥ 0.41mM); complete inhibition of sulphate reduction was observed at concentrations of DMP ≥3.28mM. From the results obtained in this batch culture study it was impossible to determine if the methanogenic bacteria had been inhibited completely. Comparing the results from the *o*-cresol and DMP batches it was noted that less DMP was needed to give the

same effect as *o*-cresol, these two compounds are similar with DMP containing an additional CH_3 group and having a lower solubility (Varhanickova *et al*., 1995).

The results obtained from the batch culture experiments gave an insight into the bacterial inhibition of the two phenolic compounds. From the results it was unclear if the individual bacterial and archaeal groups were affected directly by the compounds or if the inhibition of one bacterial group had a knock on effect.

In order to further study the effects of *o*-cresol and DMP, three stage continuous flow systems were set up. These systems separated the bacterial groups into distinct habitat domains while still allowing for overlapping activity domains. Although there was bacterial crossover amongst the vessels with methane being produced in all vessels, analysis of the results produced in Chapter 4 showed that the predominant bacterial groups and their processes could be clearly distinguished between the vessels.

The results obtained from the continuous flow systems (Chapter 4) showed complete inhibition of sulphate reduction at concentrations of 1.23mM DMP and 3.7mM *o*-cresol compared to 2.46mM DMP and 4.63mM o-cresol for methanogens. As mentioned in Section 4.8 the systems allowed for the direct effect of *o*-cresol and DMP on methanogenic and acetogenic bacteria, however to observe a direct effect on sulphate reducing bacteria the input media composition would have to contain a carbon source that could be directly utilised by the bacteria. The inhibitory concentrations observed in the system studies were lower than those recorded from the batch culture

studies. The possible reasons for the difference in inhibitory concentrations between the two studies are detailed in chapter 4.

The continuous flow multi-stage systems allowed for the study of the individual groups of bacteria and archaea. From this study it was observed that the hydrogenotrophic bacteria which had colonized the second vessel were more susceptible to *o*-cresol and DMP than the acetoclastic methanogens which existed in the third vessels of the systems; these results were only possible due to the separation of habitat domains with the overlapping of activity domains achieved by using the continuous flow systems.

Previous studies have shown that sulphate reducing bacteria are more susceptible to toxic chemicals than methanogenic bacteria (Holmes *et al.*, 2002). In the present studies it appears that this is also the case although as previously mentioned the sulphate reducing bacteria may be indirectly inhibited.

It was also discovered, throughout this project, that the time which the *o*-cresol or DMP were added to the batch cultures caused a different effect. In the experiments where the phenolic compounds were added after the onset of methanogenisis, the methanogenic archaea were more resilient to the toxic effect of the compound than if the compounds were added at the beginning of the experiment. When the compounds were added at the beginning of the experiment they affected the bacteria that produce important precursors for methanogenesis therefore reducing methane production. This could be due to lack of substrate or the correct redox conditions for methanogenesis

occurring due to inhibition of other bacterial groups. It is known that where it only takes one species of aerobic bacteria to completely mineralize a carbon source it takes all different groups of synotrophic anaerobes working together to complete the same mineralization (Smolenski and Sulfita, 1987; Parkes and Senior, 1988; McInerney *et al.*, 2009).

The batch culture studies set up to analyse the combined inhibitory effects of *o*-cresol and DMP showed that the inhibitory effect of the two compounds compound was greater than the theoretical additive effect. As mentioned in Section 5.4.3, this experiment gave results that showed the combination of the *o*-cresol and DMP exhibited a synergistic effect on the bacterial consortia including methanogenic and sulphate reducing processes. These results were not expected and if time had permitted the experiment would have been repeated. As previously mentioned these two compounds are polar narcotics and combined should have an additional concentration effect. Therefore, even if future studies showed the combined toxicity of the compounds to be additive, this can still cause a problem in the groundwater environment.

The molecular section of this thesis enabled the visualization of population shifts between the vessels of the systems and between the control system and the system perturbed with *o*-cresol and DMP. Molecular analysis of anaerobic consortia had never been done in the present laboratory and therefore it was new techniques. It was observed that these techniques could prove to be an invaluable tool in the analysis of toxic effects on bacterial consortia. As mentioned in Section 1.7, these techniques do have their drawbacks i.e. PCR not discriminating between live and dead bacteria, however using the molecular techniques coupled with the analytical

techniques could provide an excellent insight into future toxicity studies. Additional optimisation of the techniques and sequencing of the separated bacteria would have been done had time permitted.

In conclusion, the major findings of this thesis are that *o*-cresol and 2,4-dimethylphenol both proved to exhibit toxic effect on the anaerobic consortia. The inhibition of major anaerobic metabolic processes was observed. Previous studies have shown the toxicity of these compounds, and differences in toxicity have been observed. In theory DMP is more toxic than o-cresol due to its increase hydrophobicity and increased hydrogen bond donor activity (Section 1.5). The results of the current study agreed with this in all experiments, showing that DMP was considerably more toxic to the anaerobic consortia than *o*-cresol.

By utilising continuous flow multi-stage systems to separate the bacterial groups, this study enabled the analysis of specific groups, while still allowing their synotrophic interactions to take place. Therefore further study could be done on the direct effects of *o*-cresol and DMP on the bacteria processes. These studies showed that acetoclastic methanogens were more resilient to the contaminants than hydrogenotrophic methanogens, and also indicated that sulphate-reducing bacteria may have a much lower resistance to these two chemicals than the other major groups. In both the batch culture experiments and the continuous flow systems the importance of the synotrophic bacterial associations was shown. The batch results showed that by inhibiting some of the processes, other processes in the anaerobic metabolism are inhibited due to the lack of substrates or unfavourable redox conditions.

Inhibition of the natural processes of anaerobic bacterial associations found in the environment can cause the natural breakdown of carbon compounds to be inhibited, if this occurs in the natural environment it will have a knock-on effect to aquatic flora and fauna. The two compounds are known to migrate easily into the ground water environment and due to their small size may travel far, which could affect drinking water supplies (Sauter and Licha, 2002).

A major finding of this study is the observation that the combined toxicity of *o*-cresol and DMP had a synergistic effect on the anaerobic bacterial consortia. The experiments to analysis the combined toxicity of these compounds showed that they have a synergistic inhibitory effect on the bacteria. Although, as previously mentioned, repeat analysis should be carried out. None the less, these chemicals are found in the environment and are usually part of a complex mix of chemicals which are present due to chemical waste and oil spills, therefore the environmental impact of such chemical spill should be analysed. The results show that two chemicals can have a synergistic effect on the bacteria, the effect of multiple chemicals that individually may not have be present at inhibitory concentrations could cause major inhibition to the bacterial processes of the groundwater or other anaerobic environment

This study has highlighted the toxic effects of o-cresol and DMP on anaerobic consortia and the importance of the synotrophic relationships between the different bacterial groups. It is important that work in this field is carried on

to develop a better understanding of the implications of toxic chemical spills and what they do to the groundwater environment.

Chapter 7

Future Work

7.1 Future Work

Further work in this research field should include:

- Future investigations into the effects of *o*-cresol and DMP on the different bacterial/archaeal groups within the consortia, in order to possibly highlight bacterial tolerance to these toxic compounds. To do this, longer term batch culture studies should be set up and the concentrations of the chemicals they have been perturbed with should be kept constant. The cultures should be supplemented periodically with cellobiose to ensure a carbon source is present for the bacteria. This study would allow the observation of the possible development of bacterial tolerance.
- A study into the effect of o-cresol and DMP individually and combined on individual bacterial groups should be set up using pure cultures. These studies may give a better understanding of which bacterial groups within a consortium are affected more by the chemicals
- An additional study consisting of another set of batch cultures should be prepared to monitor the possible biodegradation by the bacteria/archaea of the two alkylphenolic compounds as a carbon source, the cultures should be set up in two different groups. The first group should have no alkylphenolic compound added until the cultures are active, whereas the second should have the compounds added at the start of the experiment. The concentrations of *o*-cresol and DMP should be added at sub-inhibitory levels. After the initial addition of cellobiose no further carbon source should be added and SO_4, CH_4 and VFA concentrations should be recorded as well as the

concentrations of o-cresol and DMP. This would allow observation of the possible utilisation of the alkylphenolic compounds by the bacteria.

- The batch cultures from both the above experiments should also be analysed by molecular techniques, which should be developed further and include sequencing. This may help to single out certain bacterial groups which are resistant to/or utilise the alkylphenols.
- In future studies using the multi-stage systems the input media should be changed to contain carbon sources that can be directly utilised by sulphate reducing bacteria, thus allowing for direct analysis of the effects of o-cresol and DMP on this bacterial group.
- Further studies that should be evaluated involve the effect of mixed pollutants on the activity of the bacterial consortia; it was shown during this project (Chapter 5) that *o*-cresol and DMP exhibit a synergistic effect on sulphate reducing bacteria and methanogenic bacteria/archaea when combined. The work should include further analysis into this phenomena utilising molecular tools e.g. DGGE and sequencing with possible experiments using protein mapping and other techniques to possibly identify the reasons behind the synergistic effect exhibited by the combined chemicals.
- Experiments should also be planned using more than two contaminants and different combinations of these, to further explore the effect of mixed pollutants and to develop a more environmentally relevant model. Due to the fact that in the environment bacterial consortia are exposed to a plethora of pollutants and laboratory studies on single pollutants may not give an accurate analogy of what

actually occurs in the environment. Therefore further work should be carried out on the combined toxicity of mixed pollutants.

- Future projects should investigate the effects the alkylphenols exhibit on different bacterial consortia taken from different sources for example groundwater, wastewater and consortiums extracted from ponds and rivers. These experiments would allow the effects the compounds have on consortiums with different bacterial populations to be observed. This could possibly highlight some bacterial anomalies between consortiums and allow for a more detailed study in to the growth dynamics of different consortiums.
- Experiments which vary the temperature of the multi-stage systems and batch cultures should be performed. During the current project all cultures and systems were incubated at 30°C. In future studies a range of temperatures should be used which include a temperature of 10°C to emulate groundwater conditions, this temperature should cause a slower bacterial growth rate which should be taken into account when planning the experiment to ensure sufficient time.
- Finally further work should investigate the population shifts within the consortiums and should be carried out utilising the molecular techniques including universal PCR, DGGE and molecular sequencing to develop a more comprehensive pattern of population dynamics within the consortium after pollutants stress it.

All of the further recommendations will lead to a greater understanding of this research field and possibly the development of new bioremediation technologies.

Appendix I

Appendix I: Result tables and standard deviations for Chapter 3

Tables I.1 Mean methane concentration μM/ml and standard deviations of batch cultures perturbed with *o*-cresol (n=3)

Day No.	Control Mean	STDEV	1.85mM *o*-cresol Mean	STDEV	3.7mM *o*-cresol Mean	STDEV	4.6mM *o*-cresol Mean	STDEV
0	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
17	1.72	0.540	1.48	0.292	1.32	0.011	1.61	0.046
24	5.44	1.245	4.80	1.536	3.58	0.823	3.39	0.319
31	5.43	0.089	5.34	0.484	4.59	0.173	3.36	0.531
38	4.49	0.201	3.96	0.102	3.65	0.084	2.96	0.285
47	3.86	0.562	4.49	0.074	4.35	0.154	1.77	0.896

Day No.	5.6mM *o*-cresol Mean	STDEV	7.4mM *o*-cresol Mean	STDEV	9.3mM *o*-cresol Mean	STDEV	11mM *o*-cresol Mean	STDEV
0	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
17	1.14	0.162	1.85	0.453	1.55	0.192	0.88	0.259
24	1.41	0.297	0.28	0.024	0.43	0.260	0.63	0.031
31	3.39	0.118	0.50	0.076	0.15	0.006	0.84	0.222
38	3.46	0.342	2.28	0.330	0.31	0.096	0.65	0.448
47	1.11	0.466	4.47	0.124	0.59	0.417	0.15	0.089

Tables I.2 Mean pH and standard deviations of batch cultures perturbed with *o*-cresol (n=3)

Day No.	Control Mean	STDEV	1.85mM o-cresol Mean	STDEV	3.7mM o-cresol Mean	STDEV	4.6mM o-cresol Mean	STDEV
0	7.61	0.035	7.43	0.219	7.71	0.164	7.61	0.140
5	6.56	0.017	6.63	0.052	6.52	0.066	6.47	0.059
15	6.98	0.012	6.98	0.012	6.81	0.031	6.54	0.055
30	7.72	0.012	7.56	0.189	7.76	0.121	7.64	0.060
41	7.96	0.025	7.94	0.025	7.96	0.051	7.97	0.067

Day No.	5.6mM o-cresol Mean	STDEV	7.4mM o-cresol Mean	STDEV	9.3mM o-cresol Mean	STDEV	11mM o-cresol Mean	STDEV
0	7.60	0.166	7.71	0.182	7.54	0.254	7.42	0.113
5	6.63	0.118	6.42	0.252	6.44	0.129	5.67	0.057
15	6.23	0.021	6.44	0.225	6.43	0.145	6.61	0.148
30	6.95	0.068	6.24	0.470	6.24	0.386	6.64	0.057
41	7.59	0.105	7.04	0.157	6.69	0.081	6.63	0.106

Tables I.3 Mean acetate concentration (mM) and standard deviations of batch cultures perturbed with *o*-cresol (n=3)

Day No.	Control Mean	STDEV	1.85mM o-cresol Mean	STDEV	3.7mM o-cresol Mean	STDEV	4.6mM o-cresol Mean	STDEV
0	4.30	0.100	3.90	0.100	4.20	0.100	4.03	0.0471
5	12.26	0.612	12.33	0.629	11.93	1.036	10.28	0.2975
15	14.19	0.904	15.09	0.438	12.95	0.825	11.91	0.7565
30	6.66	2.038	4.81	0.709	5.13	0.482	4.81	0.6910
41	2.26	0.649	2.12	0.189	2.16	0.893	2.07	0.0539

Day No.	5.6mM o-cresol Mean	STDEV	7.4mM o-cresol Mean	STDEV	9.3mM o-cresol Mean	STDEV	11mM o-cresol Mean	STDEV
0	4.00	0.000	3.78	0.104	4.07	0.208	4.20	0.071
5	9.35	0.932	8.60	0.646	6.10	0.348	5.05	0.185
15	11.20	2.748	10.99	0.007	10.41	0.952	8.17	0.436
30	8.45	1.882	8.86	0.063	10.80	0.296	7.76	0.337
41	1.44	0.147	5.71	0.505	10.11	2.131	7.84	0.231

Tables I.4 Mean propionate concentration (mM) and standard deviations of batch cultures perturbed with *o*-cresol (n=3)

Day No.	Control Mean	STDEV	1.85mM o-cresol Mean	STDEV	3.7mM o-cresol Mean	STDEV	4.6mM o-cresol Mean	STDEV
0	1.28	0.108	1.10	0.050	0.71	0.040	0.90	0.041
5	5.36	0.146	6.09	0.062	6.45	0.438	5.98	0.656
15	5.16	0.812	5.27	0.247	6.46	0.140	6.64	0.237
30	5.34	1.017	5.22	0.451	4.46	0.282	3.96	0.838
41	4.21	0.191	5.20	0.004	4.14	1.134	3.65	0.633

Day No.	5.6mM o-cresol Mean	STDEV	7.4mM o-cresol Mean	STDEV	9.3mM o-cresol Mean	STDEV	11mM o-cresol Mean	STDEV
0	0.70	0.019	1.21	0.027	1.02	0.100	0.79	0.027
5	5.15	1.236	5.91	0.053	3.91	0.479	0.81	0.086
15	6.34	2.106	6.50	0.006	6.56	0.365	2.47	0.289
30	5.31	0.665	6.16	0.542	6.69	0.008	2.95	0.072
41	4.85	1.880	6.49	0.011	6.42	0.803	3.24	0.314

Tables I.5 Mean iso-butyrate concentration (mM) and standard deviations of batch cultures perturbed with *o*-cresol (n=3)

Day No.	Control Mean	STDEV	1.85mM o-cresol Mean	STDEV	3.7mM o-cresol Mean	STDEV	4.6mM o-cresol Mean	STDEV
0	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
5	0.36	0.043	0.59	0.095	0.41	0.067	0.41	0.055
15	0.46	0.009	0.51	0.059	0.83	0.324	0.74	0.020
30	0.81	0.045	0.91	0.377	0.53	0.051	0.58	0.074
41	0.25	0.027	0.35	0.008	0.41	0.065	0.53	0.031

Day No.	5.6mM o-cresol Mean	STDEV	7.4mM o-cresol Mean	STDEV	9.3mM o-cresol Mean	STDEV	11mM o-cresol Mean	STDEV
0	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
5	0.36	0.022	0.48	0.069	0.17	0.082	0.07	0.011
15	0.55	0.036	0.62	0.043	0.58	0.037	0.26	0.106
30	0.70	0.055	0.38	0.017	0.67	0.024	0.42	0.135
41	0.53	0.024	0.50	0.051	0.79	0.246	0.65	0.000

Tables I.6 Mean butyrate concentration (mM) and standard deviations of batch cultures perturbed with *o*-cresol (n=3)

Day No.	Control Mean	STDEV	1.85mM o-cresol Mean	STDEV	3.7mM o-cresol Mean	STDEV	4.6mM o-cresol Mean	STDEV
0	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
5	1.14	0.017	1.43	0.136	1.25	0.206	2.34	0.297
15	1.14	0.012	1.02	0.163	1.96	0.473	2.40	0.385
30	1.41	0.123	1.26	0.259	1.40	0.072	2.32	0.261
41	0.16	0.110	0.16	0.051	0.27	0.295	1.80	0.034

Day No.	5.6mM o-cresol Mean	STDEV	7.4mM o-cresol Mean	STDEV	9.3mM o-cresol Mean	STDEV	11mM o-cresol Mean	STDEV
0	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
5	2.35	0.331	1.84	0.051	2.06	0.340	0.33	0.048
15	2.55	0.016	2.23	0.009	2.68	0.738	1.84	0.387
30	2.37	0.048	2.04	0.085	3.06	0.724	2.43	0.800
41	2.01	0.039	1.86	0.145	3.42	0.311	3.99	0.229

Tables I.7 Mean iso-valerate concentration (mM) and standard deviations of batch cultures perturbed with *o*-cresol (n=3)

Day No.	Control Mean	STDEV	1.85mM o-cresol Mean	STDEV	3.7mM o-cresol Mean	STDEV	4.6mM o-cresol Mean	STDEV
0	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
5	0.34	0.034	0.53	0.114	0.38	0.063	0.34	0.007
15	0.52	0.026	0.54	0.040	0.56	0.028	0.61	0.047
30	0.85	0.017	0.74	0.093	0.65	0.038	0.61	0.006
41	0.53	0.138	0.76	0.047	0.68	0.068	0.68	0.024

Day No.	5.6mM o-cresol Mean	STDEV	7.4mM o-cresol Mean	STDEV	9.3mM o-cresol Mean	STDEV	11mM o-cresol Mean	STDEV
0	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
5	0.34	0.007	0.43	0.102	0.16	0.053	0.07	0.004
15	0.38	0.285	0.50	0.000	0.42	0.035	0.28	0.098
30	0.52	0.246	0.55	0.023	0.63	0.076	0.51	0.079
41	0.61	0.059	0.60	0.016	0.74	0.206	0.63	0.005

Tables I.8 Mean valerate concentration (mM) and standard deviations of batch cultures perturbed with *o*-cresol (n=3)

Day No.	Control Mean	STDEV	1.85mM o-cresol Mean	STDEV	3.7mM o-cresol Mean	STDEV	4.6mM o-cresol Mean	STDEV
0	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
5	0.37	0.013	0.77	0.074	0.58	0.169	0.53	0.025
15	0.43	0.014	0.48	0.061	1.10	0.293	1.01	0.060
30	0.77	0.009	0.79	0.081	0.82	0.036	0.96	0.059
41	0.20	0.090	0.35	0.008	0.74	0.038	0.94	0.009

Day No.	5.6mM o-cresol Mean	STDEV	7.4mM o-cresol Mean	STDEV	9.3mM o-cresol Mean	STDEV	11mM o-cresol Mean
0	0.00	0.000	0.00	0.000	0.00	0.000	0.00
5	0.48	0.030	0.60	0.200	0.19	0.043	0.08
15	0.79	0.196	1.12	0.092	1.16	0.231	0.52
30	0.95	0.012	1.12	0.115	1.16	0.278	0.82
41	0.95	0.023	1.13	0.080	1.16	0.296	1.06

Tables I.9 Mean hexanoate concentration (mM) and standard deviations of batch cultures perturbed with *o*-cresol (n=3)

Day No.	Control Mean	STDEV	1.85mM o-cresol Mean	STDEV	3.7mM o-cresol Mean	STDEV	4.6mM o-cresol Mean	STDEV
0	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
5	0.08	0.035	0.09	0.006	0.15	0.075	0.09	0.006
15	0.09	0.026	0.09	0.012	0.35	0.043	0.36	0.055
30	0.36	0.021	0.31	0.037	0.23	0.034	0.28	0.084
41	0.06	0.011	0.10	0.034	0.20	0.068	0.20	0.054

Day No.	5.6mM o-cresol Mean	STDEV	7.4mM o-cresol Mean	STDEV	9.3mM o-cresol Mean	STDEV	11mM o-cresol Mean	STDEV
0	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
5	0.06	0.010	0.11	0.008	0.03	0.030	0.03	0.005
15	0.26	0.016	0.27	0.072	0.14	0.040	0.12	0.074
30	0.25	0.015	0.25	0.005	0.21	0.014	0.21	0.211
41	0.21	0.028	0.22	0.062	0.23	0.032	0.27	0.245

Tables I.10 Mean Sulphate concentration (mM) and standard deviations of batch cultures perturbed with *o*-cresol (n=3)

Day No.	Control Mean	STDEV	1.85mM o-cresol Mean	STDEV	3.7mM o-cresol Mean	STDEV	4.6mM o-cresol Mean	STDEV
1	4.40	0.132	4.30	0.063	4.35	0.166	4.21	0.067
4	3.57	0.208	3.53	0.308	3.66	0.068	3.90	0.272
6	1.95	0.369	1.88	0.402	2.94	0.619	3.78	0.136
9	1.28	0.374	1.40	0.477	2.06	0.738	3.40	0.243
11	0.51	0.051	1.18	0.634	2.25	0.254	3.40	0.268
13	0.09	0.085	0.69	0.688	1.87	0.339	3.52	0.132
16	0.00	0.000	0.29	0.256	1.07	0.344	3.50	0.079
20	0.00	0.000	0.00	0.000	0.71	0.149	3.40	0.188
22	0.00	0.000	0.00	0.000	0.23	0.024	2.91	0.219
24	0.00	0.000	0.00	0.000	0.00	0.000	2.86	0.189
27	0.00	0.000	0.00	0.000	0.00	0.000	1.96	0.653
30	0.00	0.000	0.00	0.000	0.00	0.000	0.81	0.972
33	0.00	0.000	0.00	0.000	0.00	0.000	0.71	0.792
41	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000

Tables I.11 Mean Sulphate concentration (mM) and standard deviations of batch cultures perturbed with *o*-cresol (n=3)

Day No.	5.6mM o-cresol Mean	STDEV	7.4mM o-cresol Mean	STDEV	9.3mM o-cresol Mean	STDEV	11mM o-cresol Mean	STDEV
1	4.43	0.115	4.19	0.084	4.46	0.225	4.44	0.038
4	4.32	0.206	4.24	0.183	4.40	0.219	4.22	0.073
6	3.94	0.203	4.00	0.186	4.09	0.147	4.22	0.047
9	4.03	0.124	4.23	0.214	4.12	0.159	4.18	0.022
11	3.92	0.095	4.07	0.231	3.99	0.274	4.15	0.032
13	3.99	0.049	4.03	0.173	4.10	0.214	4.36	0.016
16	4.03	0.022	3.93	0.276	4.18	0.048	4.35	0.026
20	3.99	0.063	3.88	0.204	4.34	0.167	4.29	0.188
22	4.04	0.157	4.21	0.245	4.26	0.283	4.23	0.313
24	4.10	0.157	4.02	0.153	4.27	0.169	4.24	0.093
27	4.05	0.069	4.13	0.057	4.22	0.071	4.29	0.109
30	4.00	0.006	4.12	0.016	4.20	0.105	4.27	0.063
33	3.77	0.161	4.41	0.048	4.14	0.132	4.30	0.032
41	3.51	0.246	4.15	0.073	4.11	0.198	4.27	0.109

Appendix I: Result tables and standard deviations for 2,4-Dimethylphenol batch cultures

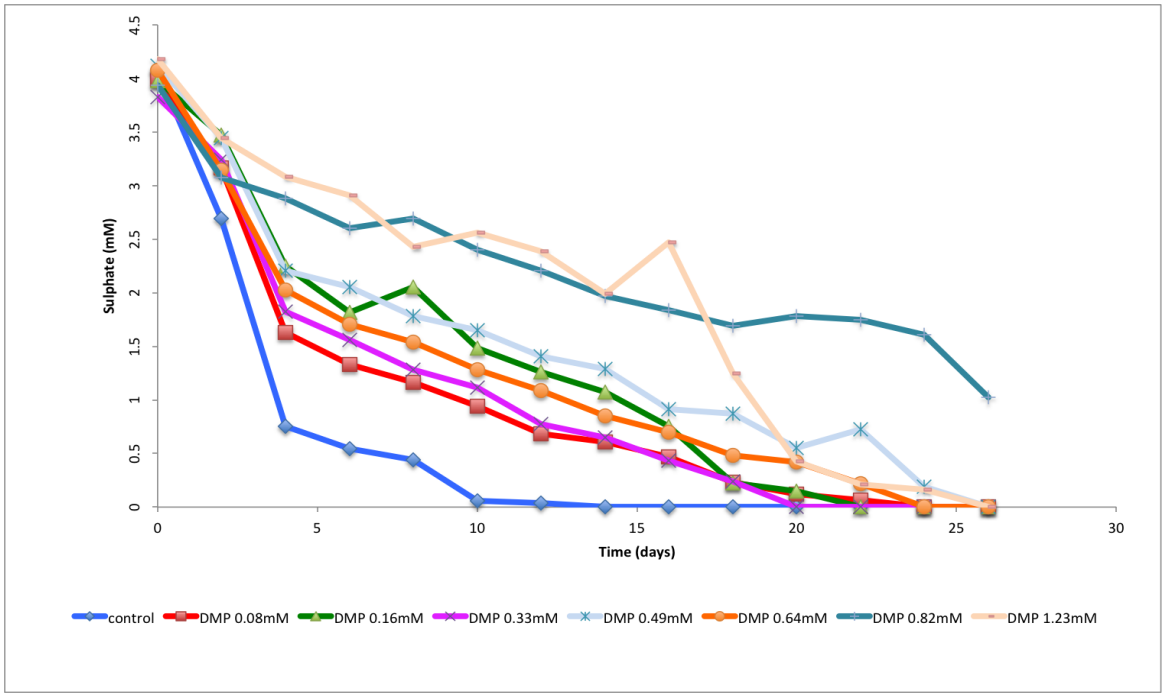


Figure I.1 Sulphate reduction versus time for batch cultures containing 2, 4-dimethylphenol (scoping experiment see Section 3.3.5)

Tables I.12 Mean pH and standard deviations of batch cultures perturbed with DMP (n=3)

DAY No.	control Mean	STDEV	DMP 0.41mM Mean	STDEV	DMP 0.82mM Mean	STDEV	DMP 1.23mM Mean	STDEV
0	0.117	7.36	0.261	7.37	0.042	7.40	0.324	7.81
6	0.006	6.89	0.076	6.68	0.038	6.71	0.040	6.52
16	0.066	7.04	0.036	6.88	0.059	6.91	0.012	6.63
28	0.095	7.83	0.078	7.13	0.099	7.20	0.103	6.61
33	0.391	7.79	0.294	7.38	0.029	7.41	0.232	6.81
41	0.359	8.05	0.046	7.88	0.093	7.85	0.030	7.45

DAY No.	control Mean	STDEV	DMP 0.41mM Mean	STDEV	DMP 0.82mM Mean	STDEV	DMP 1.23mM Mean	STDEV
0	0.015	7.49	0.081	7.71	0.121	7.59	0.310	0.015
6	0.087	6.44	0.112	6.63	0.035	6.44	0.410	0.087
16	0.064	6.52	0.021	6.58	0.010	6.75	0.297	0.064
28	0.123	6.41	0.036	6.36	0.021	5.90	0.820	0.123
33	0.057	6.49	0.133	6.64	0.066	6.57	0.301	0.057
41	0.010	7.35	0.257	7.16	0.155	6.74	0.175	0.010

Tables I.13 Mean acetate concentration (mM) and standard deviations of batch cultures perturbed with DMP (n=3)

DAY No.	control Mean	STDEV	DMP 0.41mM Mean	STDEV	DMP 0.82mM Mean	STDEV	DMP 1.23mM Mean	STDEV
0	4.48	0.751	3.67	0.858	4.81	1.202	4.39	0.375
6	17.67	0.329	17.57	0.693	15.81	3.160	17.14	0.824
16	13.79	1.400	16.91	0.391	16.80	0.841	17.44	0.345
28	9.49	0.727	10.68	0.660	14.29	1.630	14.69	0.233
33	6.84	0.189	8.33	0.808	11.81	2.007	12.95	0.421
41	2.92	0.720	5.45	0.578	9.14	2.650	9.33	1.173

DAY No.	DMP 1.64mM Mean	STDEV	DMP 2.46mM Mean	STDEV	DMP 3.28mM Mean	STDEV	DMP 4.1mM Mean	STDEV
0	5.10	0.841	5.67	1.230	3.15	1.470	3.58	0.233
6	16.10	0.665	11.10	3.953	12.92	0.970	12.03	0.639
16	16.34	0.867	13.76	0.256	11.87	0.815	12.00	0.360
28	12.69	3.427	16.16	0.864	11.92	0.544	11.62	1.212
33	11.21	0.437	13.29	1.088	11.30	0.813	11.41	0.773
41	8.53	1.153	5.98	1.311	11.02	0.023	11.12	0.431

Tables I.14 Mean propionate concentration (mM) and standard deviations of batch cultures perturbed with DMP (n=3)

DAY No.	control Mean	STDEV	DMP 0.41mM Mean	STDEV	DMP 0.82mM Mean	STDEV	DMP 1.23mM Mean	STDEV
0	1.50	0.436	1.07	0.240	1.49	0.529	1.47	0.387
6	5.37	0.498	5.27	0.561	5.23	0.163	5.02	0.275
16	4.92	1.034	5.66	0.210	5.23	0.241	5.57	0.253
28	5.47	0.243	5.26	0.086	5.30	0.556	5.04	0.377
33	5.14	0.205	5.56	0.225	5.53	0.417	5.22	0.221
41	5.22	0.521	5.48	0.262	5.52	0.061	5.69	0.537

DAY No.	DMP 1.64mM Mean	STDEV	DMP 2.46mM Mean	STDEV	DMP 3.28mM Mean	STDEV	DMP 4.1mM Mean	STDEV
0	1.28	0.156	1.61	0.499	1.02	0.290	1.02	0.070
6	4.10	0.546	3.50	0.049	2.77	0.252	2.43	0.070
16	4.23	0.197	3.60	0.213	2.57	0.239	2.23	0.321
28	4.43	1.015	3.94	0.645	2.48	0.203	1.92	0.089
33	4.63	1.146	2.97	0.382	2.55	0.182	2.00	0.031
41	4.41	0.131	3.32	0.085	2.59	0.021	2.77	0.495

Tables I.15 Mean iso-butyrate concentration (mM) and standard deviations of batch cultures perturbed with DMP (n=3)

DAY No.	control Mean	STDEV	DMP 0.41mM Mean	STDEV	DMP 0.82mM Mean	STDEV	DMP 1.23mM Mean	STDEV
0	0.46	0.234	0.30	0.059	0.46	0.250	0.35	0.051
6	0.70	0.095	0.56	0.140	0.73	0.200	0.54	0.025
16	0.80	0.382	0.82	0.149	0.77	0.186	0.73	0.156
28	0.79	0.036	0.67	0.032	0.76	0.015	0.84	0.208
33	0.66	0.015	0.69	0.038	0.65	0.099	0.86	0.072
41	0.19	0.163	0.51	0.038	0.55	0.050	0.87	0.180

DAY No.	DMP 1.64mM Mean	STDEV	DMP 2.46mM Mean	STDEV	DMP 3.28mM Mean	STDEV	DMP 4.1mM Mean	STDEV
0	0.29	0.021	0.48	0.224	0.29	0.031	0.23	0.025
6	0.57	0.079	0.48	0.092	0.49	0.010	0.50	0.006
16	0.68	0.182	0.54	0.198	0.55	0.123	0.53	0.172
28	0.88	0.278	0.72	0.145	0.63	0.075	0.67	0.106
33	0.83	0.100	0.62	0.010	0.67	0.017	0.63	0.010
41	0.83	0.125	0.71	0.103	0.73	0.061	0.81	0.049

Tables I.16 Mean butyrate concentration (mM) and standard deviations of batch cultures perturbed with DMP (n=3)

DAY No.	control Mean	STDEV	DMP 0.41mM Mean	STDEV	DMP 0.82mM Mean	STDEV	DMP 1.23mM Mean	STDEV
0	1.37	0.214	1.15	0.053	1.39	0.279	1.49	0.531
6	2.52	0.101	2.44	0.155	2.46	0.150	2.56	0.304
16	2.40	0.646	2.64	0.395	2.70	0.185	2.64	0.274
28	2.45	0.166	2.63	0.199	2.98	0.364	2.96	0.771
33	1.53	0.267	2.23	0.325	2.79	0.285	3.03	0.404
41	0.32	0.131	0.81	0.899	2.73	0.072	2.73	0.046

DAY No.	DMP 1.64mM Mean	STDEV	DMP 2.46mM Mean	STDEV	DMP 3.28mM Mean	STDEV	DMP 4.1mM Mean	STDEV
0	1.25	0.112	1.41	0.276	1.05	0.375	1.17	0.111
6	2.61	0.173	2.68	0.403	4.63	1.994	5.95	0.171
16	2.50	0.345	2.86	0.198	4.50	0.309	6.30	0.134
28	2.89	0.421	3.02	0.369	4.63	0.163	5.77	0.451
33	2.90	0.439	3.98	1.099	4.89	0.142	5.95	0.276
41	2.70	0.046	4.41	1.340	4.43	0.565	6.14	0.205

Tables I.17 Mean iso-valerate concentration (mM) and standard deviations of batch cultures perturbed with DMP (n=3)

DAY No.	control Mean	STDEV	DMP 0.41mM Mean	STDEV	DMP 0.82mM Mean	STDEV	DMP 1.23mM Mean	STDEV
0	0.51	0.219	0.34	0.035	0.61	0.225	0.50	0.136
6	0.78	0.096	0.66	0.061	0.47	0.318	0.80	0.284
16	0.65	0.006	0.87	0.137	0.81	0.061	0.91	0.026
28	0.91	0.021	0.90	0.072	0.91	0.098	0.85	0.064
33	0.91	0.021	1.02	0.250	0.90	0.038	1.03	0.178
41	0.83	0.072	0.84	0.055	0.92	0.064	1.11	0.129

DAY No.	DMP 1.64mM Mean	STDEV	DMP 2.46mM Mean	STDEV	DMP 3.28mM Mean	STDEV	DMP 4.1mM Mean	STDEV
0	0.69	0.529	0.50	0.199	0.38	0.060	0.31	0.036
6	0.63	0.035	0.71	0.120	0.64	0.076	0.65	0.031
16	0.85	0.238	0.69	0.172	0.99	0.984	0.76	0.532
28	1.03	0.291	0.83	0.031	0.80	0.085	0.69	0.049
33	0.88	0.092	0.73	0.095	0.83	0.101	0.68	0.006
41	0.91	0.055	0.87	0.111	0.76	0.934	1.00	0.240

Tables I.18 Mean valerate concentration (mM) and standard deviations of batch cultures perturbed with DMP (n=3)

DAY No.	control Mean	STDEV	DMP 0.41mM Mean	STDEV	DMP 0.82mM Mean	STDEV	DMP 1.23mM Mean	STDEV
0	0.58	0.352	0.40	0.246	0.72	0.232	0.45	0.196
6	0.58	0.169	0.78	0.291	0.60	0.111	0.64	0.026
16	0.34	0.006	0.81	0.190	0.57	0.023	0.70	0.493
28	0.55	0.102	0.46	0.006	0.56	0.066	0.61	0.049
33	0.36	0.006	0.43	0.064	0.62	0.098	0.70	0.190
41	0.41	0.099	0.43	0.040	0.43	0.015	0.46	0.129

DAY No.	DMP 1.64mM Mean	STDEV	DMP 2.46mM Mean	STDEV	DMP 3.28mM Mean	STDEV	DMP 4.1mM Mean	STDEV
0	0.44	0.156	0.54	0.314	0.42	0.144	0.35	0.068
6	1.94	0.464	2.30	1.761	2.87	0.325	5.49	0.183
16	1.45	0.500	2.06	0.175	2.45	0.061	4.02	0.873
28	1.01	0.561	1.84	1.443	1.57	0.127	2.61	0.140
33	1.07	0.266	1.00	0.057	0.87	0.053	1.66	0.533
41	1.04	0.055	1.04	0.055	1.02	0.098	1.59	0.007

Tables I.19 Mean hexanoate concentration (mM) and standard deviations of batch cultures perturbed with DMP (n=3)

DAY No.	control Mean	STDEV	DMP 0.41mM Mean	STDEV	DMP 0.82mM Mean	STDEV	DMP 1.23mM Mean	STDEV
0	0.37	0.344	0.10	0.040	0.39	0.348	0.18	0.070
6	0.08	0.133	0.09	0.076	0.07	0.075	0.25	0.055
16	0.31	0.398	0.22	0.104	0.06	0.110	0.26	0.010
28	0.18	0.064	0.09	0.012	0.14	0.049	0.23	0.042
33	0.09	0.053	0.06	0.000	0.12	0.035	0.35	0.068
41	0.21	0.133	0.10	0.015	0.11	0.025	0.27	0.167

DAY No.	DMP 1.64mM Mean	STDEV	DMP 2.46mM Mean	STDEV	DMP 3.28mM Mean	STDEV	DMP 4.1mM Mean	STDEV
0	0.08	0.010	0.31	0.314	0.09	0.051	0.07	0.012
6	0.31	0.290	0.78	0.000	0.16	0.038	0.12	0.010
16	0.46	0.165	0.73	0.079	0.68	0.092	0.54	0.193
28	0.54	0.586	0.82	0.127	1.07	0.190	0.99	0.139
33	0.59	0.042	1.09	0.096	1.03	0.071	1.07	0.180
41	0.61	0.122	1.00	0.102	1.08	0.082	1.19	0.516

Tables I.20 Mean sulphate concentration (mM) and standard deviations of batch cultures perturbed with DMP (n=3)

DAY No.	control Mean	STDEV	DMP 0.41mM Mean	STDEV	DMP 0.82mM Mean	STDEV	DMP 1.23mM Mean	STDEV
0	4.20	0.071	4.02	0.054	4.22	0.256	4.20	0.194
1	3.81	0.061	3.94	0.166	4.13	0.596	4.34	0.117
5	0.21	0.126	0.47	0.141	1.00	0.736	2.04	0.194
6	0.00	0.000	0.38	0.036	0.64	0.122	1.11	0.375
8	0.00	0.000	0.31	0.078	0.46	0.115	0.84	0.131
11	0.00	0.000	0.00	0.000	0.35	0.065	0.62	0.087
14	0.00	0.000	0.00	0.000	0.38	0.053	0.56	0.143
16	0.00	0.000	0.00	0.000	0.32	0.084	0.54	0.152
18	0.00	0.000	0.00	0.000	0.25	0.228	0.39	0.089
20	0.00	0.000	0.00	0.000	0.15	0.130	0.31	0.142
22	0.00	0.000	0.00	0.000	0.00	0.000	0.37	0.104
25	0.00	0.000	0.00	0.000	0.00	0.000	0.38	0.233
28	0.00	0.000	0.00	0.000	0.00	0.000	0.27	0.239
33	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
41	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
50	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
56	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000

Tables I.21 Mean sulphate concentration (mM) and standard deviations of batch cultures perturbed with DMP (n=3)

DAY No.	DMP 1.64mM Mean	STDEV	DMP 2.46mM Mean	STDEV	DMP 3.28mM Mean	STDEV	DMP 4.1mM Mean	STDEV
0	3.80	0.352	4.04	0.068	4.27	0.214	4.09	0.470
1	4.22	0.110	4.06	0.341	4.00	0.323	4.06	0.145
5	2.89	0.539	3.80	0.203	3.70	0.328	3.63	0.293
6	2.81	0.139	3.77	0.477	3.89	0.203	3.97	0.165
8	2.80	0.396	4.02	0.496	4.66	0.029	4.35	0.256
11	2.45	0.394	4.08	0.415	4.38	0.217	4.30	0.331
14	2.16	0.179	3.57	0.506	3.96	0.031	3.90	0.404
16	2.23	0.344	4.09	0.548	4.17	0.093	4.24	0.363
18	1.84	0.388	3.16	0.084	3.82	0.052	4.05	0.128
20	1.60	0.615	3.39	0.356	3.93	0.079	4.05	0.363
22	1.83	0.310	3.28	0.542	3.82	0.167	3.94	0.079
25	1.30	0.746	3.36	0.415	3.97	0.048	4.15	0.186
28	1.16	0.469	3.11	0.654	3.81	0.192	4.08	0.110
33	1.13	0.629	3.22	0.728	3.72	0.058	4.03	0.250
41	0.69	0.243	2.90	0.582	3.80	0.432	3.95	0.083
50	0.19	0.331	2.68	0.962	3.94	0.245	4.14	0.018
56	0.00	0.000	1.72	0.813	3.67	0.250	4.12	0.075

Tables I.22 Mean methane concentration μM/ml and standard deviations of batch cultures perturbed with DMP (n=3)

DAY No.	control Mean	STDEV	DMP 0.41mM Mean	STDEV	DMP 0.82mM Mean	STDEV	DMP 1.23mM Mean	STDEV
0	0	0	0	0	0	0	0	0
14	1.08	0.080	0.47	0.097	0.35	0.010	0.31	0.061
20	1.85	0.214	0.83	0.321	0.38	0.006	0.18	0.082
28	4.40	0.199	4.07	1.062	2.01	0.539	1.27	0.401
36	8.95	0.270	6.03	0.782	3.48	2.085	3.84	0.921
41	6.81	0.454	6.38	2.274	2.22	0.738	3.25	1.330

DAY No.	DMP 1.64mM Mean	STDEV	DMP 2.46mM Mean	STDEV	DMP 3.28mM Mean	STDEV	DMP 4.1mM Mean	STDEV
0	0	0	0	0	0	0	0	0
14	0.42	0.034	0.68	0.125	1.00	0.143	1.66	0.625
20	0.06	0.013	0.09	0.049	0.18	0.030	0.60	0.206
28	0.28	0.136	0.23	0.140	0.37	0.053	0.34	0.133
36	2.42	0.856	2.42	0.566	3.07	0.609	0.54	0.551
41	4.49	0.595	3.82	0.343	4.23	0.378	1.93	0.822

Appendix II

Appendix II: result tables for Chapter 4

Table II.1 pH for system A (n=1)

Day No.	vessel A1	vessel A2	vessel A3	Day No.	vessel A1	vessel A2	vessel A3
1	7.01	7.50		274	7.11	7.99	8.72
5	6.58	7.12	7.44	278	7.09	7.75	8.47
6	6.97	7.20	7.40	281	7.30	7.87	8.46
8	7.03	7.27	7.45	296	7.11	7.95	8.60
10	6.95	7.27	7.40	307	7.21	8.09	8.30
14	7.12	7.05	7.42	321	7.19	7.89	8.43
17	6.84	7.30	7.36	327	7.40	7.84	8.60
24	7.00	7.42	7.68	333	7.30	7.94	8.50
25	6.91	7.20	7.40	340	7.45	7.91	8.75
29	6.80	7.11	7.34	345	7.23	7.98	8.65
31	7.01	7.27	7.49	355	7.10	7.85	8.38
33	6.93	7.06	7.40	362	7.60	7.89	8.72
37	7.05	7.42	7.61	367	7.40	7.75	8.67
42	7.02	7.25	7.55	374	7.50	7.86	8.45
45	7.09	7.45	7.61	383	7.30	7.99	8.59
49	6.97	7.36	7.59	390	7.40	7.93	8.47
52	6.94	7.40	7.68	393	7.10	7.95	8.65
54	7.05	7.46	7.65	401	7.30	7.98	8.54
56	7.69	7.94	7.97	407	7.32	7.89	
61	6.98	7.70	7.99	414	7.24	7.93	
63	7.03	7.59	8.05	420	7.30	7.91	
67	7.05	7.50	7.91	440	7.30	7.80	
69	7.20	7.54	7.80	446	7.31	7.92	
73	6.86	7.50	7.83	456	7.25	7.85	
76	6.97	7.36	7.81	462	7.35	7.93	
83	7.00	7.40	8.10	465	7.27	7.90	
90	7.10	7.54	7.90				
102	7.13	7.60	8.23				
113	7.09	7.30	8.12				
121	7.40	7.35	7.99				
129	7.20	7.80	8.43				
137	7.10	7.50	8.57				
145	7.30	7.70	8.23				
150	7.04	7.67	8.45				
161	7.10	7.93	8.50				
168	7.12	7.58	8.20				
174	7.30	8.07	8.32				
185	7.40	7.90	8.23				
190	7.54	7.84	8.56				
200	7.30	7.79	8.34				

Table II.2 pH for system B (n=1)

Day No.	vessel B1	vessel B2	vessel B3	Day No.	vessel B1	vessel B2	vessel B3
1	7.16	7.60		274	7.30	7.83	8.27
5	6.66	7.38	7.29	278	7.55	7.99	8.67
6	6.83	7.26	7.44	281	7.44	7.85	8.26
8	7.12	7.48	7.49	296	7.42	7.64	7.99
10	6.93	7.25	7.40	307	7.60	7.95	8.46
14	6.96	7.27	7.43	321	7.46	7.81	8.34
17	6.74	7.17	7.39	327	7.50	7.94	8.00
24	7.00	7.43	7.64	333	7.50	8.00	8.27
25	6.72	7.16	7.43	340	7.15	7.80	8.50
29	6.57	7.06	7.42	345	7.12	7.75	8.27
31	6.83	7.37	7.78	355	6.85	7.54	8.20
33	6.88	7.18	7.61	362	7.15	7.67	8.21
37	7.20	7.43	7.60	367	7.10	7.70	8.30
42	6.84	7.39	7.65	374	7.20	7.60	8.21
45	6.75	7.42	7.59	383	7.02	7.50	8.30
49	6.89	7.45	7.62	390	7.00	7.56	8.60
52	6.78	7.38	7.65	393	6.90	7.70	8.30
54	6.60	7.17	7.68	401	7.30	7.90	8.40
56	7.07	7.54	7.85	407	7.20	7.50	8.50
61	6.85	7.24	7.63	414	7.10	7.80	8.30
63	6.84	7.25	7.56	420	7.30	7.60	8.40
67	6.98	7.32	7.59	440	7.40	7.70	8.40
69	6.99	7.63	7.80	446	7.20	7.50	8.50
73	6.70	7.45	7.85	456	7.20	7.60	8.30
76	6.80	7.46	7.89	462	7.30	7.60	8.20
83	6.90	7.50	7.90	465	7.10	7.70	8.40
90	7.21	7.40	8.10				
102	7.12	7.60	7.90				
113	6.99	7.56	8.30				
121	7.23	7.43	8.20				
129	7.21	7.70	8.40				
137	7.32	7.30	8.30				
145	7.45	7.60	8.30				
150	7.49	7.70	8.20				
161	7.38	7.50	8.30				
168	7.54	7.80	8.40				
174	7.37	7.90	8.20				
185	7.50	7.77	8.20				
190	7.40	7.87	8.50				
200	7.20	7.65	8.30				

VFA samples were run with standards in between each run.

Table II.3 Acetate concentrations (mM) for system A (n=1)

Day No.	vessel A1	vessel A2	vessel A3	Day No.	vessel A1	vessel A2	vessel A3
1	11.96	8.70	0.00	108	12.66	12.40	7.15
3	10.79	9.37	10.43	123	12.63	9.57	6.80
4	9.46	9.71	11.95	125	8.97	9.97	6.82
5	9.71	10.48	9.80	132	12.90	10.26	7.38
6	7.38	8.50	9.84	139	14.96	13.94	8.93
7	8.79	8.86	10.09	152	12.23	11.96	7.37
8	8.55	8.25	9.26	168	11.45	10.71	6.81
9	8.51	9.74	10.05	181	13.40	11.19	6.77
12	9.39	11.83	10.10	195	11.52	10.66	6.21
13	9.82	10.24	10.57	204	10.25	12.52	5.28
15	11.61	9.90	9.79	216	11.65	11.98	7.60
17	10.41	9.72	11.33	231	12.53	12.05	8.61
19	11.85	10.33	8.98	237	7.76	7.68	6.48
21	11.33	10.57	9.72	252	8.62	7.80	7.30
23	12.80	11.79	10.98	261	11.27	7.45	5.62
25	11.59	10.80	10.91	270	11.13	7.00	6.23
27	11.36	11.49	9.07	275	10.18	7.07	7.30
29	13.40	12.47	10.37	279	12.41	8.85	8.67
31	12.07	11.49	7.71	282	12.38	9.69	7.50
33	11.90	10.72	8.16	288	8.09	7.10	5.80
35	13.06	11.59	8.68	297	11.59	7.35	5.24
37	12.48	10.40	7.49	321	11.86	8.60	4.96
39	11.36	11.56	8.19	329	10.73	7.98	3.86
41	11.06	10.51	8.26	339	9.72	7.75	3.56
43	12.17	11.99	8.64	350	10.19	8.30	3.93
45	10.35	9.94	8.34	356	10.05	7.26	4.14
48	10.93	10.37	7.58	363	8.55	7.75	4.08
52	12.92	9.96	6.65	366	11.76	7.53	4.42
54	11.24	8.45	5.33	372	10.62	8.34	4.95
56	13.33	8.98	2.19	378	9.82	7.34	4.62
59	10.62	8.49	3.98	399	7.88	8.60	4.50
61	11.11	6.53	3.14	407	9.60	8.76	4.90
66	13.63	12.66	6.24	421	9.20	8.54	5.63
68	9.38	7.94	4.67	440	9.80	8.35	6.52
72	11.50	7.74	4.06	446	10.10		
75	8.45	7.69	4.26	456	9.80		
81	8.16	8.56	4.63	462	9.90		
83	10.98	10.44	6.94	465	10.00		
87	8.91	10.09	5.95				
102	12.98	8.64	7.72				

Table II.4 Acetate concentrations (mM) for system B (n=1)

Day No.	vessel B1	vessel B2	vessel B3	Day No.	vessel B1	vessel B2	vessel B3
1	10.08	9.71	0.00	108	10.55	9.37	5.00
3	7.75	8.15	6.49	123	9.78	7.93	7.22
4	7.75	7.90	8.17	125	9.16	10.95	7.29
5	6.70	7.12	7.34	132	11.37	8.49	7.40
6	7.21	7.30	8.22	139	9.44	8.91	8.42
7	7.41	6.52	7.93	152	10.08	10.28	8.33
8	7.09	7.05	8.17	168	9.59	10.60	8.20
9	8.27	7.29	7.85	181	10.10	8.74	4.88
12	8.36	10.42	9.22	195	10.68	8.35	5.55
13	7.63	8.62	8.51	204	11.00	8.75	6.58
15	7.34	8.24	7.90	216	10.65	8.25	7.96
17	7.69	7.23	8.11	231	10.83	8.10	8.91
19	7.48	7.41	7.60	237	8.01	8.32	7.25
21	8.94	7.90	8.15	252	7.32	7.20	7.54
23	9.43	8.68	7.74	261	10.54	8.65	7.30
25	9.58	8.80	6.72	270	9.89	7.81	6.40
27	11.53	8.81	5.76	275	10.23	8.22	7.29
29	10.87	8.97	5.19	279	11.99	10.63	8.90
31	7.68	6.19	3.75	282	10.45	10.74	9.76
33	8.73	6.31	3.97	288	8.30	7.50	6.30
35	8.24	6.77	3.71	297	7.65	7.29	8.47
37	8.62	7.81	3.61	321	10.14	6.10	5.03
39	10.32	8.90	4.85	329	10.05	7.87	6.94
41	11.00	9.23	4.50	339	10.67	10.28	8.07
43	8.27	7.41	4.37	350	11.18	8.63	7.11
45	8.97	7.92	4.25	356	9.85	9.81	8.05
48	10.24	9.17	4.13	363	12.71	10.84	7.36
52	12.03	9.06	5.07	364	10.40	10.61	8.00
54	11.78	9.33	4.92	372	8.59	8.99	5.55
56	10.15	9.96	5.76	378	8.31	9.22	5.90
59	8.68	8.05	3.80	399	6.04	9.30	4.83
61	8.78	5.69	4.68	406	6.06	8.76	3.30
66	13.22	10.32	5.20	421	7.04	9.32	4.30
68	12.09	6.97	3.00	440	9.70	8.90	3.07
72	11.90	6.61	2.67	446	10.30		
75	9.75	6.33	2.62	456	9.70		
81	11.11	7.83	3.19	462	10.00		
83	12.01	9.79	4.52	465	10.20		
87	9.60	8.15	5.23				
102	12.10	9.68	5.25				

Table II.5 Propionate concentrations (mM) for system A (n=1)

Day No.	vessel A1	vessel A2	vessel A3	Day No.	vessel A1	vessel A2	vessel A3
1	2.89	1.17		108	5.59	3.13	1.31
3	2.50	1.17	0.93	123	3.79	2.11	1.79
4	1.91	1.04	0.71	125	1.56	1.73	1.81
5	2.31	1.06	0.92	132	3.72	1.87	1.87
6	0.79	0.72	0.71	139	4.37	2.76	2.10
7	1.73	1.04	0.85	152	3.46	2.55	1.90
8	1.72	0.96	0.78	168	2.24	1.79	1.43
9	1.27	0.90	0.90	181	3.82	2.33	1.87
12	2.58	1.32	1.24	195	3.65	2.86	1.88
13	1.73	1.16	0.94	204	3.25	2.66	1.52
15	2.41	1.45	0.96	216	3.76	2.65	1.57
17	2.44	1.21	0.84	231	2.39	2.34	1.71
19	1.77	1.00	0.94	237	1.43	1.43	1.53
21	1.95	1.06	0.93	252	1.66	0.46	0.09
23	2.77	1.36	0.95	261	1.39	1.65	1.77
25	1.72	0.97	0.88	270	0.88	0.31	1.07
27	1.66	0.93	0.94	275	2.10	1.01	1.00
29	2.37	1.36	0.84	279	2.87	2.05	1.34
31	1.87	0.85	0.85	282	3.99	2.58	1.94
33	1.43	0.93	0.82	288	1.08	1.79	1.76
35	2.43	1.10	0.88	297	2.09	1.32	1.38
37	1.61	0.90	0.92	321	2.72	1.28	1.48
39	1.80	0.98	1.05	329	2.81	1.81	1.31
41	0.92	0.90	0.80	339	1.88	1.71	0.56
43	2.22	1.18	1.15	350	2.69	1.13	1.31
45	1.51	0.97	1.01	356	1.71	1.72	1.54
48	1.15	0.97	0.88	363	1.66	2.30	1.80
52	2.20	1.25	0.94	364	3.50	2.15	1.53
54	1.31	1.04	0.92	372	3.41	3.08	1.83
56	3.06	1.53	0.87	378	1.37	2.45	1.78
59	1.80	1.06	1.02	399	4.67	3.62	2.41
61	1.07	1.00	0.83	406	1.65	2.44	3.25
66	3.59	1.67	1.38	421	1.96	3.88	2.17
68	1.28	1.23	1.02	440	1.73	1.84	1.62
72	1.36	0.98	0.90				
75	0.81	1.05	1.05				
81	0.90	0.96	0.92				
83	2.38	1.25	1.11				
87	0.76	0.83	0.87				
102	2.28	1.03	0.94				

Table II.6 Propionate concentrations (mM) for system B (n=1)

Day No.	vessel B1	vessel B2	vessel B3	Day No.	vessel B1	vessel B2	vessel B3
1	0.85	0.75		108	1.47	0.16	0.81
3	0.65	0.98	0.58	123	1.45	0.72	1.05
4	0.96	0.85	0.76	125	1.40	1.13	1.06
5	0.59	0.66	0.69	132	1.82	0.64	1.11
6	0.76	0.64	0.64	139	1.97	1.44	1.60
7	0.64	0.65	0.62	152	2.22	1.58	1.26
8	0.66	0.64	0.70	168	1.44	1.17	1.07
9	0.92	0.65	0.70	181	2.40	1.18	0.92
12	1.13	1.04	0.79	195	1.95	1.26	1.62
13	0.84	0.79	0.85	204	2.22	1.43	0.99
15	0.91	0.82	0.73	216	2.56	1.46	1.64
17	0.78	0.80	0.75	231	2.74	1.68	1.92
19	0.80	0.81	0.82	237	1.75	1.58	1.64
21	0.81	0.81	0.81	252	0.49	0.15	1.07
23	0.70	0.84	0.80	261	1.32	1.09	1.26
25	0.99	0.77	0.78	270	2.01	1.00	1.26
27	0.93	0.90	0.86	275	1.91	1.00	1.11
29	0.88	1.07	0.92	279	1.98	1.41	1.07
31	1.12	0.88	0.93	282	1.81	1.40	1.15
33	0.67	0.88	0.86	288	1.54	0.99	0.84
35	0.73	0.72	0.83	297	0.67	1.07	0.97
37	0.74	0.82	0.75	321	2.24	0.81	1.01
39	0.97	0.89	0.88	329	1.99	1.28	1.15
41	0.82	0.87	0.87	339	2.98	2.83	1.70
43	0.86	0.76	0.86	350	3.00	1.96	1.54
45	0.73	0.79	0.82	356	3.64	2.87	2.45
48	0.79	0.81	0.85	363	3.62	3.20	2.57
52	0.68	0.82	0.82	364	3.06	2.92	2.90
54	0.82	0.85	0.82	372	3.05	2.23	2.44
56	0.88	0.91	0.85	378	3.12	2.09	2.46
59	0.94	0.83	0.78	399	4.54	4.08	2.78
61	0.82	0.75	0.81	406	1.60	1.22	1.63
66	0.94	0.94	0.84	421	2.30	1.63	1.47
68	0.72	0.68	0.63				
72	0.71	0.69	0.79				
75	0.79	0.71	0.70				
81	0.75	0.75	0.67				
83	1.28	1.01	0.72				
87	0.88	0.85	0.88				
102	0.94	0.89	0.83				

Table II.7 Iso-butyrate concentrations (mM) for system A (n=1)

Day No.	vessel A1	vessel A2	vessel A3	Day No.	vessel A1	vessel A2	vessel A3
1	1.11	0.39		108	1.82	0.68	0.16
3	0.83	0.30	0.20	123	1.07	0.51	0.24
4	0.46	0.24	0.12	125	0.32	0.21	0.28
5	0.56	0.25	0.24	132	0.82	0.26	0.42
6	0.16	0.19	0.10	139	1.17	0.51	0.34
7	0.48	0.27	0.20	152	0.91	0.47	0.30
8	0.40	0.23	0.17	168	0.30	0.26	0.16
9	0.31	0.20	0.20	181	0.94	0.41	0.20
12	0.55	0.31	0.35	195	1.46	0.42	0.19
13	0.45	0.28	0.22	204	0.85	0.43	0.27
15	0.69	0.47	0.26	216	0.92	0.54	0.24
17	0.78	0.35	0.22	231	1.10	0.53	0.28
19	0.46	0.24	0.27	237	0.70	0.24	0.13
21	0.58	0.26	0.22	252	0.60	0.07	0.05
23	0.82	0.30	0.19	261	0.76	0.46	0.25
25	0.30	0.17	0.17	270	0.18	0.04	0.05
27	0.37	0.14	0.21	275	0.21	0.13	0.11
29	0.51	0.30	0.14	279	0.42	0.33	0.18
31	0.37	0.14	0.15	282	0.92	0.38	0.34
33	0.25	0.17	0.13	288	0.44	0.23	0.22
35	0.64	0.20	0.15	297	0.55	0.24	0.15
37	0.35	0.14	0.15	321	1.15	0.41	0.37
39	0.48	0.17	0.21	329	0.11	0.16	0.07
41	0.13	0.15	0.10	339	0.77	0.49	0.14
43	0.47	0.22	0.23	350	0.28	0.19	0.13
45	0.33	0.16	0.15	356	0.71	0.36	0.20
48	0.19	0.15	0.10	363	0.11	0.66	4.67
52	0.28	0.23	0.12	364	0.21	0.14	0.07
54	0.47	0.13	0.10	372	0.72	0.68	0.25
56	0.99	0.30	0.06	378	0.28	0.45	0.13
59	0.39	0.07	0.14	399	0.97	0.49	0.27
61	0.09	0.07	0.04	406	0.28	0.26	0.72
66	1.12	0.24	0.25	421	0.56	1.05	0.37
68	0.12	0.17	0.11	440	0.15	0.19	0.13
72	0.09	0.06	0.04				
75	0.08	0.08	0.04				
81	0.11	0.14	0.05				
83	0.93	0.31	0.16				
87	0.16	0.17	0.10				
102	0.14	0.16	0.20				

Table II.8 Iso-butyrate concentrations (mM) for system B (n=1)

Day No.	vessel B1	vessel B2	vessel B3	Day No.	vessel B1	vessel B2	vessel B3
1	0.19	0.14		108	0.23	0.15	0.09
3	0.14	0.36	0.12	123	0.17	0.15	0.13
4	0.34	0.21	0.20	125	0.20	0.11	0.69
5	0.15	0.15	0.20	132	0.36	0.21	0.26
6	0.29	0.20	0.19	139	0.46	0.21	0.30
7	0.15	0.16	0.21	152	0.24	0.22	0.19
8	0.14	0.12	0.26	168	0.16	0.12	0.16
9	0.27	0.10	0.23	181	0.19	0.10	0.08
12	0.27	0.22	0.29	195	0.16	0.16	0.15
13	0.20	0.20	0.39	204	0.25	0.16	0.15
15	0.24	0.17	0.27	216	0.46	0.13	0.16
17	0.22	0.19	0.24	231	0.32	0.20	0.16
19	0.21	0.19	0.23	237	0.20	0.11	0.11
21	0.16	0.19	0.21	252	0.17	0.05	0.07
23	0.11	0.17	0.16	261	0.32	0.11	0.10
25	0.20	0.12	0.31	270	0.41	0.11	0.17
27	0.08	0.14	0.14	275	0.31	0.08	0.12
29	0.08	0.19	0.14	279	0.26	0.16	0.10
31	0.20	0.14	0.15	282	0.30	0.19	0.12
33	0.05	0.13	0.09	288	0.28	0.10	0.11
35	0.12	0.10	0.08	297	0.20	0.14	0.12
37	0.10	0.11	0.07	321	0.46	0.24	0.22
39	0.19	0.14	0.09	329	0.13	0.09	0.09
41	0.06	0.08	0.05	339	0.38	0.77	0.30
43	0.16	0.09	0.07	350	0.30	0.22	0.19
45	0.09	0.10	0.06	356	0.48	0.27	0.21
48	0.07	0.08	0.05	363	0.31	0.26	0.23
52	0.06	0.13	0.07	364	0.19	0.20	0.18
54	0.09	0.10	0.05	372	0.44	0.23	0.25
56	0.10	0.10	0.07	378	0.32	0.06	0.10
59	0.15	0.12	0.06	399	0.65	0.53	0.16
61	0.08	0.10	0.07	406	0.25	0.15	0.15
66	0.12	0.14	0.06	421	0.25	0.16	0.23
68	0.09	0.06	0.03	440	0.20	0.17	0.09
72	0.06	0.06	0.04				
75	0.08	0.06	0.05				
81	0.07	0.06	0.03				
83	0.18	0.15	0.04				
87	0.12	0.11	0.09				
102	0.13	0.18	0.10				

Table II.9 Butyrate concentrations (mM) for system A (n=1)

Day No.	vessel A1	vessel A2	vessel A3	Day No.	vessel A1	vessel A2	vessel A3
1	2.76	1.44		108	2.42	0.98	0.24
3	3.15	1.67	1.24	123	1.76	0.71	0.28
4	3.70	2.00	1.20	125	0.24	0.15	0.25
5	4.20	2.35	1.57	132	1.65	0.30	0.48
6	3.25	2.63	1.45	139	1.81	0.70	0.41
7	3.25	2.88	1.93	152	1.52	0.67	0.33
8	3.20	2.86	2.06	163	0.76	0.38	0.18
9	2.77	2.83	2.23	181	1.35	0.54	0.23
12	2.69	2.78	2.55	195	1.46	0.54	0.25
13	2.51	2.32	2.38	204	1.25	0.43	0.28
15	3.02	2.21	2.17	216	0.95	0.52	0.24
17	2.37	1.24	1.67	232	1.36	0.52	0.24
19	1.60	0.63	1.21	237	0.94	0.27	0.14
21	1.92	0.64	0.44	252	1.09	0.16	0.07
23	2.34	0.79	0.22	261	0.92	0.54	0.31
25	1.22	0.57	0.18	272	0.59	0.07	0.03
27	0.89	0.36	0.24	275	1.90	0.41	0.17
29	1.32	0.61	0.14	279	0.97	0.49	0.26
31	1.38	0.49	0.17	282	1.63	0.53	0.40
33	1.03	0.49	0.16	288	0.66	0.29	0.27
35	1.54	0.55	0.20	297	5.44	0.44	0.16
37	1.57	0.61	0.26	321	1.37	0.48	0.42
39	1.81	0.69	0.32	329	0.30	0.12	0.05
41	1.67	0.64	0.14	339	0.95	0.52	0.20
43	2.73	0.94	0.34	350	0.20	0.19	0.11
45	2.68	1.10	0.32	356	0.91	0.38	0.18
48	1.60	0.24	0.06	363	0.07	0.82	0.43
52	1.20	0.24	0.14	364	0.36	0.08	0.06
54	0.69	0.10	0.10	372	0.98	1.05	0.28
56	1.44	0.43	0.10	378	0.20	0.29	0.12
59	0.86	0.09	0.18	399	1.13	0.50	0.32
61	0.18	0.08	0.06	406	0.32	0.22	1.00
66	2.05	0.40	0.37	421	0.52	1.32	0.48
68	0.28	0.30	0.12	440	0.23	0.18	0.10
72	0.44	0.06	0.03				
75	0.94	0.23	0.05				
81	1.37	0.31	0.06				
83	2.36	0.51	0.20				
87	1.66	0.38	0.14				
102	0.73	0.15	0.62				

Table II.10 Butyrate concentrations (mM) for system B (n=1)

Day No.	vessel B1	vessel B2	vessel B3	Day No.	vessel B1	vessel B2	vessel B3
1	3.69	2.67		108	0.61	0.18	0.09
3	3.84	3.24	2.85	123	0.37	0.16	0.16
4	3.84	3.70	2.62	125	0.32	0.17	0.24
5	4.18	3.99	3.03	132	0.49	0.22	0.25
6	4.09	3.90	3.17	139	1.64	0.45	0.44
7	4.16	3.84	3.04	152	0.28	0.21	0.17
8	3.82	3.69	3.25	163	1.00	0.12	0.13
9	3.93	3.60	3.11	181	0.33	0.06	0.09
12	3.95	2.65	3.49	195	0.33	0.15	0.09
13	3.59	3.08	3.70	204	0.46	0.09	0.15
15	3.18	2.42	2.79	216	0.23	0.10	0.13
17	3.10	1.61	1.86	232	0.37	0.15	0.13
19	3.03	1.14	0.68	237	0.13	0.04	0.09
21	2.69	0.63	0.17	252	1.36	0.25	0.09
23	2.59	0.43	0.09	261	0.21	0.07	0.00
25	2.09	0.31	0.07	272	0.61	0.15	0.18
27	0.44	0.15	0.09	275	1.49	0.09	0.10
29	1.24	0.36	0.14	279	0.28	0.12	0.07
31	2.96	1.06	0.15	282	0.31	0.15	0.08
33	2.93	0.64	0.08	288	0.19	0.09	0.08
35	3.44	0.53	0.08	297	1.59	0.16	0.13
37	2.39	0.44	0.08	321	0.44	0.25	0.22
39	0.88	0.20	0.11	329	0.52	0.11	0.06
41	0.90	0.16	0.05	339	0.48	0.94	0.30
43	2.84	0.68	0.13	350	0.28	0.19	0.11
45	2.63	0.80	0.11	356	0.39	0.21	0.14
48	1.58	0.34	0.06	363	0.58	0.23	0.15
52	0.12	0.13	0.06	364	0.45	0.17	0.10
54	0.35	0.17	0.05	372	0.22	0.22	0.27
56	1.16	0.39	0.12	378	0.09	0.11	0.11
59	2.84	0.89	0.12	399	0.72	0.58	0.19
61	1.75	0.45	0.04	406	0.45	0.27	0.15
66	0.18	0.11	0.06	421	0.59	0.49	0.35
68	0.09	0.06	0.04	440	0.47	0.17	0.08
72	0.33	0.06	0.04				
75	2.03	0.61	0.08				
81	0.81	0.07	0.03				
83	0.29	0.19	0.06				
87	2.41	0.37	0.13				
102	0.57	0.20	0.10				

Table II.11 iso-valerate concentrations (mM) for system A (n=1)

Day No.	vessel A1	vessel A2	vessel A3	Day No.	vessel A1	vessel A2	vessel A3
1	0.90	0.27		108	1.28	0.45	0.16
3	0.62	0.18	0.13	123	0.81	0.36	0.15
4	0.34	0.15	0.06	125	0.24	0.13	0.23
5	0.42	0.13	0.21	132	0.64	0.18	0.36
6	0.09	0.08	0.04	139	0.93	0.37	0.25
7	0.34	0.17	0.15	152	0.82	0.38	0.25
8	0.33	0.13	0.13	168	0.23	0.19	0.11
9	0.24	0.10	0.10	181	0.73	0.34	0.18
12	0.40	0.23	0.21	195	0.78	0.39	0.17
13	0.37	0.17	0.11	204	0.85	0.37	0.16
15	0.51	0.33	0.15	216	0.99	0.30	0.18
17	0.62	0.24	0.15	231	0.95	0.38	0.20
19	0.36	0.16	0.17	237	0.68	0.18	0.13
21	0.51	0.21	0.15	252	0.28	0.09	0.07
23	0.64	0.21	0.13	261	0.74	0.40	0.22
25	0.21	0.11	0.10	270	0.16	0.03	0.06
27	0.32	0.07	0.14	275	0.12	0.08	0.09
29	0.37	0.23	0.08	279	0.11	0.25	0.14
31	0.30	0.09	0.00	282	0.78	0.27	0.21
33	0.21	0.11	0.08	288	0.41	0.18	0.16
35	0.54	0.14	0.09	297	0.49	0.18	0.11
37	0.31	0.09	0.10	321	0.96	0.35	0.34
39	0.39	0.11	0.15	329	0.06	0.07	0.05
41	0.31	0.10	0.06	339	0.64	0.36	0.15
43	0.32	0.14	0.17	350	0.17	0.16	0.08
45	0.27	0.10	0.10	356	0.61	0.03	0.15
48	0.32	0.09	0.07	363	0.06	0.06	0.26
52	0.50	0.20	0.12	364	0.10	0.06	0.06
54	0.36	0.09	0.09	372	0.49	0.58	0.19
56	0.75	0.26	0.08	378	0.23	0.11	0.09
59	0.31	0.08	0.13	399	0.94	0.39	0.20
61	0.05	0.08	0.06	406	0.22	0.17	0.68
66	0.91	0.16	0.23	421	0.51	0.89	0.29
68	0.10	0.14	0.13	440	0.47	0.45	0.40
72	0.06	0.03	0.05				
75	0.04	0.05	0.04				
81	0.08	0.09	0.05				
83	0.88	0.26	0.15				
87	0.12	0.13	0.10				
102	0.69	0.12	0.13				

Table II.12 Iso-valerate concentrations (mM) for system B (n=1)

Day No.	vessel B1	vessel B2	vessel B3	Day No.	vessel B1	vessel B2	vessel B3
1	0.11	0.09		108	0.17	0.10	0.08
3	0.08	0.36	0.06	123	0.11	0.09	0.10
4	0.30	0.15	0.13	125	0.16	0.08	0.17
5	0.10	0.12	0.14	132	0.34	0.15	0.21
6	0.27	0.11	0.09	139	0.40	0.15	0.26
7	0.11	0.13	0.09	152	0.19	0.14	0.15
8	0.11	0.09	0.15	168	0.18	0.09	0.11
9	0.20	0.07	0.08	181	0.08	0.06	0.07
12	0.21	0.19	0.11	195	0.23	0.13	0.08
13	0.15	0.11	0.19	204	0.58	0.19	0.90
15	0.23	0.12	0.13	216	0.94	0.14	0.05
17	0.19	0.14	0.11	231	0.98	0.14	0.09
19	0.18	0.17	0.14	237	0.94	0.05	0.09
21	0.15	0.16	0.15	252	0.21	0.08	0.07
23	0.08	0.15	0.11	261	0.13	0.06	0.05
25	0.18	0.09	0.06	270	0.32	0.08	0.16
27	0.05	0.11	0.10	275	0.14	0.08	0.07
29	0.06	0.15	0.11	279	0.08	0.07	0.07
31	0.00	0.00	0.15	282	0.11	0.11	0.07
33	0.06	0.08	0.07	288	0.07	0.09	0.06
35	0.11	0.08	0.07	297	0.19	0.09	0.07
37	0.08	0.09	0.06	321	0.32	0.18	0.18
39	0.15	0.11	0.10	329	0.03	0.03	0.04
41	0.03	0.05	0.06	339	0.16	0.56	0.78
43	0.14	0.07	0.07	350	0.12	0.16	0.07
45	0.07	0.07	0.07	356	0.11	0.10	0.11
48	0.06	0.06	0.07	363	0.22	0.12	0.10
52	0.04	0.09	0.08	364	0.05	0.08	0.07
54	0.06	0.08	0.07	372	0.20	0.10	0.25
56	0.08	0.08	0.07	378	0.07	0.10	0.10
59	0.14	0.08	0.05	399	0.55	0.43	0.14
61	0.06	0.07	0.04	406	0.22	0.27	0.10
66	0.12	0.10	0.08	421	0.16	0.12	0.23
68	0.08	0.05	0.04	440	0.10	0.12	0.19
72	0.05	0.05	0.06				
75	0.07	0.05	0.04				
81	0.05	0.06	0.05				
83	0.14	0.14	0.05				
87	0.10	0.09	0.08				
102	0.09	0.13	0.08				

Table II.13 Valerate concentrations (mM) for system A (n=1)

Day No.	vessel A1	vessel A2	vessel A3	Day No.	vessel A1	vessel A2	vessel A3
1	1.16	0.38		108	1.72	0.63	0.20
3	0.86	0.25	0.18	123	1.14	0.54	0.23
4	0.49	0.19	0.07	125	0.37	0.19	0.31
5	0.61	0.14	0.26	132	0.92	0.26	0.44
6	0.08	0.06	0.03	139	1.32	0.53	0.35
7	0.46	0.18	0.17	152	1.03	0.49	0.32
8	0.46	0.15	0.12	168	0.26	0.23	0.14
9	0.32	0.08	0.10	181	1.00	0.42	0.20
12	0.53	0.28	0.25	195	1.11	0.45	0.32
13	0.48	0.16	0.08	204	1.01	0.52	0.65
15	0.67	0.36	0.14	216	0.99	0.50	0.21
17	0.83	0.28	0.17	231	1.17	0.43	2.22
19	0.49	0.15	0.17	237	0.86	0.27	0.14
21	0.68	0.23	0.12	252	0.03	0.03	0.04
23	0.87	0.27	0.12	261	0.87	0.48	0.23
25	0.31	0.12	0.08	270	0.05	0.06	0.03
27	0.45	0.08	0.16	275	0.09	0.06	1.30
29	0.56	0.30	0.08	279	0.13	0.31	0.15
31	0.43	0.09	0.10	282	1.03	0.33	0.30
33	0.29	0.13	0.08	288	0.54	0.28	0.21
35	0.73	0.18	0.09	297	0.54	0.22	0.11
37	0.40	0.09	0.11	321	1.31	0.46	0.43
39	0.51	0.12	0.18	329	0.56	0.07	0.04
41	0.10	0.08	0.04	339	0.91	0.49	0.19
43	0.45	0.17	0.20	350	0.17	0.19	0.10
45	0.36	0.09	0.10	356	0.94	0.42	0.19
48	0.08	0.07	0.05	363	0.05	0.82	0.35
52	0.78	0.26	0.14	364	0.11	0.05	1.17
54	0.44	0.09	0.09	372	0.79	0.81	2.13
56	0.93	0.31	0.08	378	0.32	1.14	0.10
59	0.43	0.13	0.16	399	1.04	0.42	0.39
61	1.85	0.06	0.07	406	0.23	0.16	0.91
66	1.18	0.20	0.29	421	0.69	1.15	0.38
68	1.90	0.17	0.00	440	0.55	0.02	0.87
72	0.05	0.03	0.05				
75	0.03	0.04	0.02				
81	0.11	0.11	0.05				
83	1.14	0.31	0.17				
87	0.10	0.13	0.10				
102	0.50	0.12	0.15				

Table II.14 Valerate concentrations (mM) for system B (n=1)

Day No.	vessel B1	vessel B2	vessel B3	Day No.	vessel B1	vessel B2	vessel B3
1	0.15	0.12		108	0.17	0.13	0.08
3	0.09	0.45	0.08	123	0.67	0.15	0.12
4	0.37	0.19	0.16	125	0.20	0.10	0.22
5	0.07	0.11	0.16	132	0.58	0.19	0.25
6	0.29	0.09	0.09	139	0.51	0.20	0.32
7	0.08	0.12	0.09	152	0.21	0.18	0.17
8	0.08	0.06	0.17	168	0.12	0.10	0.13
9	0.21	0.37	0.05	181	0.71	0.04	0.05
12	0.21	0.11	0.08	195	0.62	0.07	0.10
13	0.14	0.09	0.17	204	0.67	0.05	0.12
15	0.20	0.09	0.11	216	0.13	0.12	0.06
17	0.17	0.10	0.08	231	0.17	0.10	0.10
19	0.15	0.13	0.11	237	0.10	0.04	0.05
21	0.11	0.12	0.10	252	1.24	0.02	0.04
23	0.05	0.10	0.05	261	0.47	0.06	0.02
25	0.19	0.04	0.02	270	0.37	0.09	0.15
27	0.03	0.07	0.08	275	0.08	0.06	0.06
29	0.59	0.14	0.96	279	1.22	0.11	0.05
31	0.00	0.00	0.18	282	0.13	0.11	0.06
33	0.02	0.08	0.03	288	0.09	0.06	0.06
35	0.10	0.06	0.03	297	0.14	0.09	0.07
37	0.07	0.06	0.04	321	1.40	0.21	0.21
39	0.19	0.11	0.09	329	0.87	0.04	0.02
41	0.03	0.03	0.04	339	1.08	0.84	0.25
43	0.15	0.06	0.06	350	0.66	0.16	0.07
45	0.08	0.07	0.05	356	0.10	0.12	0.09
48	0.06	0.06	0.03	363	0.20	0.13	0.09
52	0.03	0.08	0.06	364	0.05	0.06	0.04
54	0.06	0.08	0.04	372	0.20	0.09	0.24
56	0.08	0.08	0.07	378	0.14	0.08	0.10
59	0.14	0.08	0.05	399	1.42	0.43	0.15
61	0.03	0.05	0.02	406	0.26	0.11	0.09
66	0.11	0.10	0.06	421	0.15	0.12	0.31
68	0.07	0.00	0.02	440	0.13	0.10	0.22
72	0.02	0.03	0.06				
75	0.04	0.02	0.02				
81	0.03	0.04	0.03				
83	0.16	0.16	0.04				
87	0.08	0.10	0.08				
102	0.09	0.12	0.07				

Table II.15 Hexanoate concentrations (mM) for system A (n=1)

Day No.	vessel A1	vessel A2	vessel A3	Day No.	vessel A1	vessel A2	vessel A3
1	1.02	0.33		108	2.33	0.48	0.17
3	0.80	0.20	0.14	123	1.09	0.49	0.22
4	0.47	0.18	0.06	125	0.24	0.15	0.25
5	0.58	0.12	0.20	132	0.83	0.25	0.43
6	0.02	0.00	0.02	139	1.20	0.48	0.31
7	0.45	0.10	0.14	152	0.85	0.42	0.29
8	0.42	0.12	0.10	168	0.22	0.19	0.12
9	0.29	0.07	0.07	181	0.91	0.36	0.18
12	0.52	0.22	0.21	195	0.84	0.37	0.22
13	0.48	0.16	0.07	204	0.65	0.33	0.13
15	0.59	0.26	0.11	216	0.95	0.35	0.10
17	0.72	0.20	0.10	231	1.21	0.35	0.00
19	0.41	0.12	0.09	237	0.95	0.23	0.12
21	0.60	0.21	0.12	252	0.01	0.01	0.03
23	0.74	0.23	0.10	261	0.91	0.40	0.18
25	0.27	0.09	0.02	270	0.00	0.00	0.03
27	0.38	0.06	0.08	275	0.03	0.03	0.04
29	0.53	0.25	0.05	279	0.13	0.25	0.12
31	0.42	0.09	0.00	282	1.06	0.23	0.23
33	0.26	0.10	0.05	288	0.51	0.18	0.16
35	0.65	0.14	0.08	297	0.43	0.16	0.06
37	0.36	0.06	0.07	321	1.14	0.37	0.37
39	0.48	0.11	0.14	329	0.02	0.06	0.03
41	0.10	0.05	0.02	339	0.85	0.39	0.15
43	0.46	0.14	0.16	350	0.14	0.16	0.07
45	0.26	0.05	0.05	356	0.92	0.37	0.21
48	0.02	0.00	0.00	363	0.04	0.79	0.29
52	0.73	0.17	0.08	364	0.03	0.05	0.00
54	0.41	0.10	0.07	372	0.65	0.71	0.24
56	0.82	0.25	0.05	378	0.22	0.06	0.08
59	0.40	0.06	0.12	399	1.32	0.47	0.27
61	0.01	0.01	0.00	406	0.23	0.16	1.05
66	1.10	0.19	0.24	421	0.48	1.19	0.37
68	0.01	0.11	0.10	440	0.39	0.35	0.33
72	0.01	0.01	0.01				
75	0.03	0.01	0.01				
81	0.02	0.05	0.03				
83	1.11	0.30	0.17				
87	0.09	0.11	0.09				
102	0.48	0.09	0.00				

Table II.16 Hexanoate concentrations (mM) for system B (n=1)

Day No.	vessel B1	vessel B2	vessel B3	Day No.	vessel B1	vessel B2	vessel B3
1	0.08	0.06		108	0.17	0.17	0.06
3	0.07	0.41	0.07	123	0.16	0.15	0.14
4	0.33	0.15	0.14	125	0.17	0.07	0.18
5	0.08	0.01	0.13	132	0.26	0.20	0.23
6	0.28	0.07	0.02	139	0.46	0.17	0.29
7	0.07	0.18	0.02	152	0.17	0.16	0.16
8	0.07	0.06	0.10	168	0.09	0.09	0.11
9	0.18	0.03	0.04	181	0.09	0.04	0.05
12	0.19	0.81	0.08	195	0.09	0.03	0.03
13	0.12	0.07	0.14	204	0.07	0.04	0.05
15	0.19	0.08	0.09	216	0.09	0.07	0.04
17	0.16	0.09	0.07	231	0.18	0.07	0.02
19	0.13	0.11	0.09	237	0.07	0.02	0.04
21	0.10	0.11	0.09	252	0.04	0.01	0.02
23	0.04	0.09	0.04	261	0.10	0.04	0.01
25	0.15	0.02	0.01	270	0.42	0.10	0.14
27	0.03	0.05	0.06	275	0.05	0.03	0.04
29	0.06	0.11	0.08	279	0.07	0.04	0.04
31	0.00	0.05	0.01	282	0.12	0.09	0.05
33	0.05	0.02	0.02	288	0.06	0.00	0.03
35	0.09	0.04	0.03	297	0.16	0.07	0.05
37	0.05	0.03	0.00	321	0.30	0.18	0.17
39	0.15	0.07	0.07	329	0.03	0.02	0.02
41	0.02	0.02	0.01	339	0.17	0.78	0.22
43	0.12	0.05	0.05	350	0.08	0.14	0.05
45	0.04	0.03	0.03	356	0.11	0.10	0.05
48	0.02	0.01	0.01	363	0.18	0.09	0.07
52	0.03	0.06	0.00	364	0.04	0.00	0.00
54	0.06	0.05	0.03	372	0.17	0.09	0.19
56	0.07	0.07	0.05	378	0.09	0.05	0.07
59	0.12	0.05	0.00	399	0.41	0.46	0.19
61	0.01	0.01	0.00	406	0.30	0.12	0.08
66	0.10	0.08	0.05	421	0.18	0.13	0.27
68	0.05	0.03	0.01	440	0.13	0.10	0.24
72	0.05	0.02	0.01				
75	0.02	0.01	0.02				
81	0.01	0.01	0.01				
83	0.15	0.15	0.05				
87	0.07	0.09	0.08				
102	0.08	0.00	0.06				

Tables II.17 – II.22 show the results for the dissolved methane from systems A and B

Table II.17: Dissolved methane (mmol/L) and standard deviations for vessel A1 (n=3)

Day No.	Mean peak area	A1 (mmol/L)	Std Dev	Day No.	Mean peak area	A1 (mmol/L)	Std Dev
6	7.98	0.24	0.222	200	6.15	0.21	0.255
9	7.17	0.20	0.396	207	7.26	0.29	0.324
11	12.52	0.31	0.401	222	7.85	0.3	0.215
13	15.13	0.30	0.257	231	3.73	0.14	0.234
15	10.86	0.26	0.392	240	3.57	0.15	0.234
17	18.64	0.44	0.390	248	4.13	0.17	0.012
19	15.30	0.34	0.191	254	6.20	0.19	0.331
21	11.48	0.32	0.230	259	7.28	0.23	0.571
25	10.45	0.27	0.823	266	4.64	0.12	0.903
27	11.17	0.28	0.344	270	3.27	0.11	0.049
30	11.84	0.37	0.413	272	6.84	0.38	0.255
32	12.47	0.35	0.420	274	5.47	0.15	0.220
34	12.80	0.33	0.042	276	9.35	0.33	0.150
37	11.21	0.32	0.237	279	6.81	0.24	0.095
42	9.40	0.27	0.896	284	3.67	0.11	0.033
45	12.36	0.28	0.560	288	3.53	0.11	0.312
47	5.93	0.20	0.144	311	5.67	0.21	0.137
55	12.31	0.40	0.262	322	5.35	0.18	0.391
60	9.63	0.31	0.168	328	3.30	0.12	0.356
67	8.73	0.24	0.314	331	2.13	0.11	0.170
75	12.96	0.42	0.352	343	3.07	0.10	0.133
79	5.21	0.21	0.207	350	5.12	0.13	0.258
95	20.05	0.67	0.052	364	5.53	0.15	0.115
103	11.96	0.45	0.361	366	5.53	0.18	0.115
108	7.15	0.29	0.235	371	5.78	0.16	0.166
125	9.48	0.24	0.145	373	4.31	0.13	0.239
131	6.84	0.17	0.186	376	3.99	0.10	0.225
136	9.68	0.31	0.536	386	4.16	0.13	0.049
144	8.03	0.27	0.103	396	4.50	0.11	0.175
156	9.18	0.24	0.244	407	5.04	0.13	0.232
168	9.44	0.33	0.206	409	4.73	0.13	0.210
181	6.14	0.22	1.712	414	6.20	0.17	0.290
185	6.08	0.22	0.154	421	5.09	0.15	0.326
191	6.29	0.25	0.147				

Table II.18: Dissolved methane (mmol/L) and standard deviations for vessel A2 (n=3)

Day No.	Mean peak area	A2 (mmol/L)	Std Dev	Day No.	Mean peak area	A2 (mmol/L)	Std Dev
6	1.53	0.05	0.184	200	9.15	0.33	0.125
9	1.81	0.05	0.146	207	9.45	0.37	0.237
11	4.25	0.11	0.307	222	10.34	0.76	0.127
13	10.82	0.21	0.123	231	11.87	0.44	0.305
15	12.64	0.31	0.593	240	9.55	0.42	0.348
17	15.92	0.37	0.618	248	8.23	0.35	0.126
19	17.12	0.38	0.256	254	6.92	0.21	0.140
21	12.55	0.35	0.513	259	15.84	0.51	0.826
25	15.80	0.41	0.445	266	15.62	0.40	0.309
27	15.71	0.40	0.457	270	6.43	0.22	0.531
30	16.70	0.52	0.528	272	10.07	0.56	0.302
32	16.95	0.48	0.531	274	14.46	0.40	0.278
34	13.93	0.36	0.188	276	15.05	0.54	0.507
37	17.43	0.50	0.389	279	15.68	0.55	0.866
42	14.55	0.42	0.940	284	13.26	0.40	0.513
45	18.78	0.43	0.323	288	11.91	0.39	0.330
47	17.53	0.60	1.127	311	11.71	0.43	0.875
55	15.62	0.50	0.553	322	10.07	0.33	0.521
60	13.02	0.42	0.147	328	10.83	0.38	0.448
67	18.81	0.51	0.497	331	12.63	0.66	0.516
75	16.62	0.54	0.244	343	8.15	0.26	0.256
79	12.47	0.50	0.144	350	10.47	0.27	0.293
95	9.87	0.33	0.254	364	7.80	0.21	0.235
103	12.00	0.46	0.327	366	6.49	0.21	0.031
108	12.39	0.50	0.716	371	8.60	0.23	0.270
125	15.48	0.39	0.168	373	2.75	0.08	0.812
131	11.47	0.28	0.389	376	2.81	0.07	0.379
136	12.73	0.40	0.632	386	3.40	0.11	0.082
144	12.41	0.41	0.408	396	3.73	0.09	0.259
156	14.41	0.38	0.366	407	4.51	0.11	0.355
168	14.39	0.51	0.306	409	0.86	0.02	0.724
181	8.86	0.32	0.242	414	0.23	0.01	0.234
185	9.66	0.36	0.397	421	0.00	0.00	0.000
191	9.68	0.35	0.255				

Table II.19: Dissolved methane (mmol/L) and standard deviations for vessel A3 (n=3)

Day No.	Mean peak area	A3 (mmol/L)	Std Dev	Day No.	Mean peak area	A3 (mmol/L)	Std Dev
6	0.59	0.02	0.271	200	10.24	0.36	0.255
9	0.57	0.02	0.132	207	10.54	0.35	0.146
11	0.83	0.02	0.065	222	10.72	0.37	0.146
13	1.48	0.03	0.077	231	10.64	0.39	0.188
15	2.05	0.05	0.082	240	9.34	0.35	0.321
17	7.05	0.17	0.158	248	8.42	0.25	0.212
19	6.85	0.15	0.093	254	4.50	0.14	0.149
21	3.30	0.09	0.103	259	1.72	0.05	0.255
25	6.83	0.18	0.443	266	2.25	0.06	0.025
27	13.08	0.33	0.539	270	4.65	0.16	1.749
30	18.37	0.57	0.723	272	2.22	0.12	0.430
32	16.14	0.46	0.284	274	1.18	0.03	0.167
34	11.87	0.31	0.200	276	1.20	0.04	0.150
37	16.31	0.47	1.148	279	1.41	0.05	0.269
42	19.18	0.55	0.718	284	1.74	0.05	0.309
45	20.03	0.46	0.418	288	2.76	0.09	0.146
47	19.24	0.66	0.489	311	1.56	0.06	0.514
55	13.97	0.45	0.436	322	2.51	0.08	0.180
60	8.03	0.26	0.462	328	11.67	0.41	0.379
67	12.48	0.40	0.314	331	2.85	0.15	0.210
75	12.51	0.50	0.185	343	3.41	0.11	0.101
79	11.13	0.44	0.359	350	3.80	0.10	0.300
95	14.65	0.49	0.145	364	4.40	0.12	0.170
103	15.71	0.60	0.304	366	4.29	0.14	0.236
108	13.63	0.55	0.472	371	7.38	0.20	0.552
125	5.57	0.14	0.034	373	3.66	0.11	0.125
131	19.33	0.48	0.373	376	6.05	0.15	0.172
136	16.32	0.52	0.762	386	5.07	0.16	0.049
144	7.22	0.24	0.258	396	5.01	0.13	0.110
156	7.28	0.19	0.258	407	5.15	0.13	0.207
168	5.65	0.20	0.154	409	2.88	0.08	0.378
181	9.90	0.35	0.051	414	3.32	0.09	0.340
185	10.73	0.39	0.173	421	4.92	0.15	0.280
191	10.12	0.32	0.129				

Table II.20: Dissolved methane (mmol/L) and standard deviations for vessel B1 (n=3)

Day No.	Mean peak area	B1 (mmol/L)	Std Dev	Day No.	Mean peak area	B1 (mmol/L)	Std Dev
6	6.25	0.19	0.213	200	16.26	0.62	0.452
9	7.84	0.22	0.359	207	15.99	0.61	0.322
11	6.07	0.15	0.076	222	16.58	0.64	0.126
13	8.49	0.17	0.509	231	15.56	0.65	0.234
15	11.27	0.27	0.141	240	17.52	0.65	0.276
17	11.98	0.28	0.265	248	15.38	0.63	0.123
19	12.35	0.28	0.395	254	14.78	0.60	0.287
21	11.17	0.31	0.173	259	16.52	0.51	0.256
25	8.29	0.22	0.473	266	14.72	0.47	2.148
27	18.62	0.47	0.596	270	11.25	0.29	0.107
30	7.87	0.25	0.422	272	10.55	0.36	0.241
32	10.20	0.29	0.365	274	16.56	0.93	1.026
34	6.50	0.17	0.021	276	17.83	0.50	1.565
37	11.21	0.32	0.165	279	14.31	0.51	0.494
42	7.90	0.23	0.240	284	21.42	0.75	0.660
45	10.19	0.23	0.125	288	9.25	0.28	0.243
47	10.86	0.37	0.042	311	12.24	0.40	0.507
55	16.97	0.45	0.179	322	15.29	0.57	0.163
60	13.19	0.43	0.306	328	24.63	0.81	0.199
67	7.84	0.25	0.191	331	5.91	0.21	0.275
75	7.00	0.19	0.117	343	2.55	0.13	0.223
79	5.81	0.19	0.425	350	4.27	0.14	0.197
95	8.38	0.33	0.119	364	4.65	0.12	0.236
103	7.50	0.25	0.206	366	3.73	0.10	1.293
108	18.46	0.70	0.301	371	3.40	0.11	0.357
125	7.03	0.28	0.061	373	4.57	0.12	0.074
131	9.02	0.23	0.169	376	3.00	0.09	0.025
136	15.39	0.38	1.235	386	3.71	0.09	0.570
144	11.20	0.36	0.727	396	3.14	0.10	0.333
156	6.76	0.22	0.055	407	2.90	0.07	0.087
168	12.71	0.33	0.273	409	2.80	0.07	0.211
181	9.48	0.33	0.145	414	3.44	0.09	0.201
185	18.80	0.67	0.406	421	6.00	0.16	0.067
191	17.29	0.64	0.462				

Table II.21: Dissolved methane (mmol/L) and standard deviations for vessel B2 (n=3)

Day No.	Mean peak area	B2 (mmol/L)	Std Dev	Day No.	Mean peak area	B2 (mmol/L)	Std Dev
6	1.71	0.05	0.065	200	15.24	0.59	0.256
9	2.48	0.07	0.288	207	13.25	0.44	0.266
11	4.82	0.12	0.229	222	14.29	0.46	0.235
13	9.08	0.18	0.108	231	10.34	0.43	0.127
15	12.70	0.31	0.379	240	11.41	0.42	0.304
17	16.06	0.38	0.553	248	10.43	0.39	0.298
19	19.66	0.44	0.081	254	9.23	0.30	0.137
21	13.42	0.38	0.465	259	8.25	0.25	0.164
25	17.56	0.46	0.745	266	11.44	0.36	0.853
27	22.08	0.56	0.425	270	11.12	0.29	0.479
30	18.47	0.58	0.797	272	9.72	0.33	0.235
32	28.45	0.80	0.566	274	10.54	0.59	0.430
34	25.61	0.66	0.615	276	11.43	0.32	0.277
37	25.49	0.74	0.108	279	12.56	0.45	0.372
42	17.22	0.50	0.959	284	12.95	0.46	0.356
45	30.15	0.69	0.632	288	13.74	0.42	0.107
47	25.14	0.86	0.384	311	10.42	0.34	0.079
55	19.36	0.51	0.155	322	11.34	0.42	0.190
60	17.55	0.57	0.308	328	6.02	0.20	0.333
67	16.96	0.54	0.475	331	11.81	0.41	0.404
75	21.68	0.59	0.167	343	11.22	0.58	0.235
79	19.63	0.63	0.487	350	13.88	0.44	0.226
95	16.77	0.67	0.223	364	12.78	0.33	0.333
103	17.25	0.58	0.500	366	3.41	0.09	0.416
108	13.96	0.53	0.238	371	3.26	0.11	0.225
125	12.09	0.48	0.240	373	1.92	0.05	0.369
131	16.14	0.40	0.121	376	1.65	0.05	0.118
136	12.99	0.32	0.272	386	1.91	0.05	0.166
144	11.80	0.37	0.289	396	1.58	0.05	0.442
156	11.14	0.37	0.495	407	1.26	0.03	0.251
168	11.97	0.31	0.369	409	1.97	0.05	0.100
181	15.48	0.55	0.168	414	0.01	0.00	0.017
185	13.01	0.47	0.631	421	0.11	0.00	0.165
191	15.52	0.57	0.392				

Table II.22: Dissolved methane (mmol/L) and standard deviations for vessel B3 (n=3)

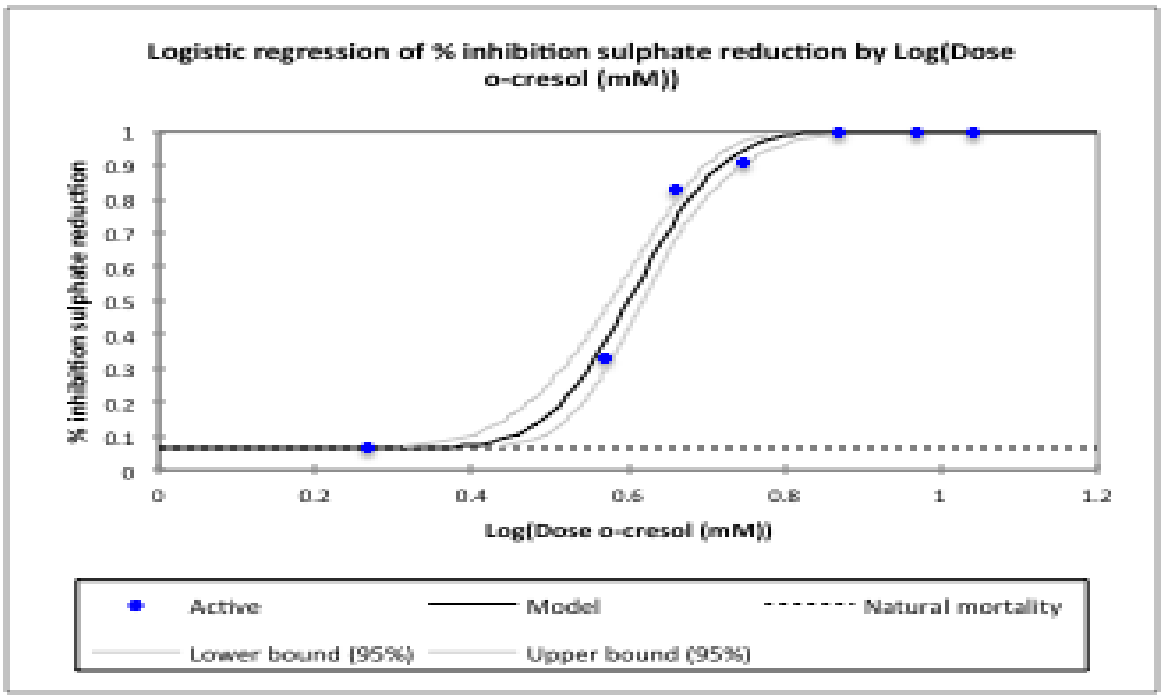
Day No.	Mean peak area	B3 (mmol/L)	Std Dev	Day No.	Mean peak area	B3 (mmol/L)	Std Dev
6	1.21	0.04	0.055	200	5.21	0.20	0.215
9	1.07	0.03	0.133	207	5.02	0.19	0.127
11	2.88	0.07	0.100	222	4.99	0.18	0.365
13	6.81	0.14	0.108	231	6.29	0.35	0.198
15	12.20	0.30	0.292	240	9.80	0.36	0.381
17	18.64	0.44	0.962	248	10.54	0.21	0.272
19	17.93	0.40	0.261	254	10.64	0.16	0.361
21	14.99	0.42	0.243	259	2.87	0.09	0.216
25	15.92	0.41	0.526	266	5.14	0.16	0.111
27	25.49	0.65	0.387	270	6.36	0.16	0.141
30	27.51	0.86	0.788	272	8.66	0.30	0.217
32	26.21	0.74	0.464	274	1.50	0.08	0.800
34	25.08	0.65	0.544	276	1.45	0.04	0.078
37	25.76	0.75	0.170	279	1.65	0.06	0.131
42	23.96	0.69	0.540	284	2.53	0.09	0.232
45	33.76	0.77	1.010	288	2.70	0.08	0.025
47	20.73	0.71	0.468	311	3.66	0.12	0.286
55	27.20	0.72	0.571	322	4.41	0.16	0.226
60	24.59	0.79	0.295	328	0.93	0.03	0.226
67	22.23	0.71	0.936	331	4.39	0.15	0.315
75	19.31	0.52	0.033	343	0.01	0.00	0.010
79	13.71	0.44	0.043	350	1.14	0.04	0.010
95	16.42	0.65	0.502	364	4.78	0.12	0.082
103	9.46	0.32	0.095	366	11.22	0.30	0.433
108	10.08	0.38	0.115	371	10.57	0.34	0.245
125	15.59	0.62	0.118	373	16.53	0.44	1.677
131	4.92	0.12	0.075	376	13.49	0.40	0.188
136	8.56	0.21	0.205	386	16.65	0.42	0.356
144	7.41	0.24	0.271	396	13.90	0.44	0.758
156	14.20	0.47	0.203	407	14.83	0.38	0.365
168	4.24	0.11	0.142	409	15.06	0.38	0.495
181	5.57	0.20	0.034	414	12.30	0.34	2.315
185	10.03	0.36	0.017	421	17.94	0.49	0.270
191	5.56	0.20	0.175				

Appendix III

Appendix III result tables for Chapter 5

O-cresol statistical analysis data

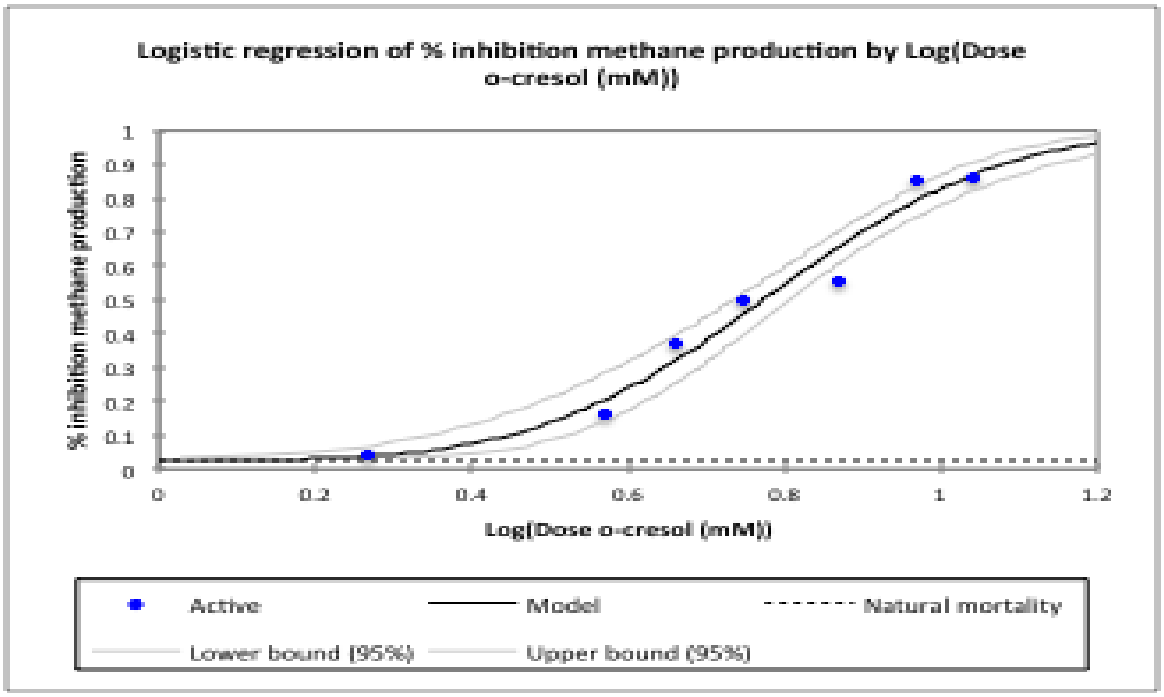
Sulphate reduction



Probability analysis with the fitted model (Variable % inhibition sulphate reduction):

Probability	Dose o-cresol (mM)	Lower bound 95%	Upper bound 95%
0.01			
0.05			
0.10	2.825	2.473	3.085
0.20	3.268	2.970	3.487
0.30	3.538	3.279	3.733
0.40	3.767	3.539	3.944
0.50	3.985	3.785	4.150
0.60	4.210	4.031	4.372
0.70	4.460	4.292	4.636
0.80	4.768	4.589	4.989
0.90	5.226	4.995	5.558
0.95	5.635	5.335	6.094
0.99	6.486	6.013	7.263

Methane Production

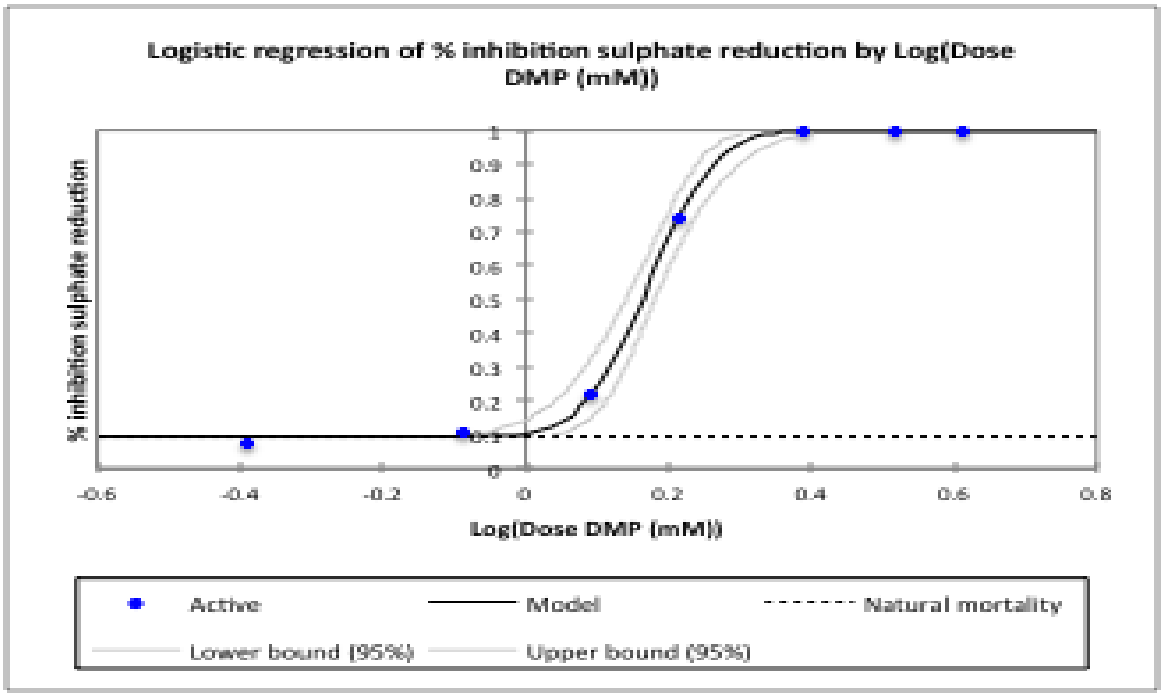


Probability analysis with the fitted model (Variable % inhibition methane production):

Probability	Dose o-cresol (mM)	Lower bound 95%	Upper bound 95%
0.01			
0.05	2.121	1.564	2.614
0.10	2.801	2.203	3.308
0.20	3.682	3.079	4.180
0.30	4.425	3.845	4.902
0.40	5.156	4.613	5.615
0.50	5.937	5.431	6.394
0.60	6.828	6.336	7.330
0.70	7.924	7.381	8.575
0.80	9.425	8.698	10.435
0.90	11.978	10.770	13.872
0.95	14.593	12.776	17.630
0.99	21.123	17.507	27.753

2,4-Dimethylphenol statistical analysis data

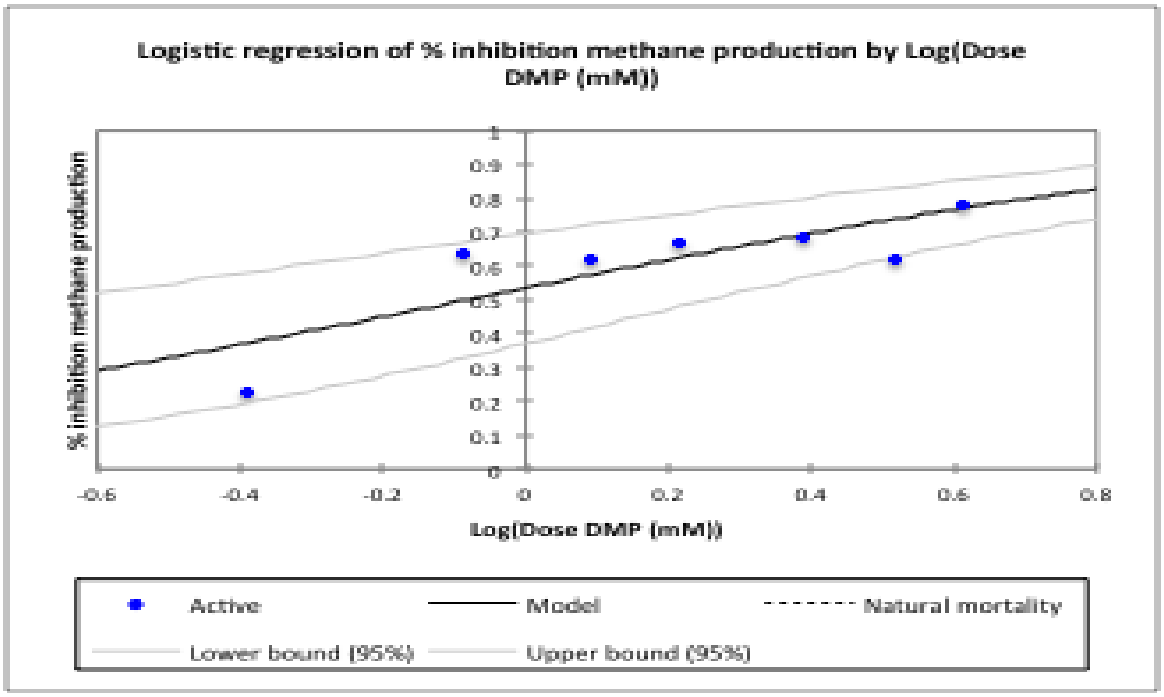
Sulphate Reduction



Probability analysis with the fitted model (Variable % inhibition sulphate reduction):

Probability	Dose DMP (mM)	Lower bound 95%	Upper bound 95%
0.01			
0.05			
0.10	0.979	0.817	1.088
0.20	1.217	1.098	1.296
0.30	1.311	1.213	1.380
0.40	1.387	1.303	1.450
0.50	1.456	1.384	1.518
0.60	1.526	1.461	1.592
0.70	1.603	1.538	1.682
0.80	1.696	1.623	1.801
0.90	1.832	1.737	1.990
0.95	1.951	1.831	2.164
0.99	2.195	2.015	2.538

Methane Production



Probability analysis with the fitted model (Variable % inhibition methane production):

Probability	Dose DMP (mM)	Lower bound 95%	Upper bound 95%
0.01	0.005	0.000	0.036
0.05	0.024	0.001	0.110
0.10	0.052	0.004	0.200
0.20	0.134	0.015	0.417
0.30	0.266	0.042	0.716
0.40	0.477	0.100	1.146
0.50	0.825	0.220	1.806
0.60	1.426	0.475	2.903
0.70	2.560	1.043	5.006
0.80	5.077	2.436	10.190
0.90	13.126	6.713	32.131
0.95	28.759	13.812	93.098
0.99	125.253	46.698	784.174

Table III.1 Mean sulphate concentration (mg/mL) for batch cultures contaminated with combinations of o-cresol and 2,4DMP to the theoretical inhibition value of 60%

Day No.	control	60% (1.15mM DMP, 0mM o-cresol)	60% (0mM DMP,4.25 mM o-cresol)	60% (1.39mM DMP, 2.8mM o-cresol)	60% (1.39mM DMP, 2.8mM o-cresol)
1	497.71	547.82	557.43	523.45	491.41
14	0	350.46	357.32	440.83	454.27
% sulphate inhibition	0	63.97	64.1	84.22	92.44
standard deviation	0	1.415	2.612	3.411	5.112

Table III.2 Mean sulphate concentration (mg/mL) for batch cultures contaminated with combinations of o-cresol and 2,4DMP to the theoretical inhibition value of 80%

Day No.	control	80% (1.81mM DMP ,0mM o-cresol)	80% (0mM DMP, 4.5mM o-cresol)	80% (1.39mM DMP, 3.8mM o-cresol)	80% (1.15mM DMP, 4.25mM o-cresol)	80% (1.53mM DMP, 2.8mM o-cresol)
1	497.71	527.69	519.24	508.96	475.46	493.039
14	0.00	388.07	366.34	463.31	472.17	475.14
% sulphate inhibition	0.00	73.54	70.55	91.03	99.31	96.37
standard deviation	0.000	4.282	7.921	8.164	3.021	10.13

Table III.3 Mean cumulative methane (μM/ml) from triplicate batch cultures contaminated with combinations of o-cresol and 2,4DMP to the theoretical inhibition value of 60%

Day No.	control	60% (1.15mM DMP, 0mM o-cresol)	60% (0mM DMP,4.25 mM o-cresol)	60% (1.39mM DMP, 2.8mM o-cresol)	60% (1.39mM DMP, 2.8mM o-cresol)
0	0	0	0	0	0
21	10.30	10.10	9.57	11.14	9.97
29	16.87	17.62	17.11	17.28	15.32
34	28.73	28.04	26.69	24.00	23.13
36	34.26	33.66	32.55	28.87	28.44
44	41.52	39.89	37.23	33.22	33.09
54	47.19	43.62	40.04	33.86	34.59
65	57.02	52.06	46.51	35.38	36.11
71	59.89	55.29	52.08	36.93	36.84
Std Dev.	2.33	2.28	2.93	1.34	0.57

Table IV.4 Mean cumulative methane (μM/ml) from triplicate batch cultures contaminated with combinations of o-cresol and 2,4DMP to the theoretical inhibition value of 80%

Day No.	control	80% (1.81mM DMP ,0mM o-cresol)	80% (0mM DMP, 4.5mM o-cresol)	80% (1.39mM DMP, 3.8mM o-cresol)	80% (1.15mM DMP, 4.25mM o-cresol)
0	0	0.00	0.00	0.00	0.00
21	10.30	10.47	9.95	10.20	10.64
29	16.87	16.46	17.09	17.62	18.58
34	28.73	23.88	27.16	26.02	26.25
36	34.26	29.68	31.75	30.58	30.37
44	41.52	34.87	35.60	33.28	33.90
54	47.19	36.76	37.23	33.62	34.40
65	57.02	41.33	41.53	34.26	35.39
71	59.89	46.20	46.83	34.98	36.11
Std Dev.	2.33	1.14	1.50	0.72	2.71

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